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Symposium on The Antimetabolites—Their Modes of Action and Therapeutic Implications*

An antimetabolite is an inactive compound sufficiently similar in structure to an essential metabolite so as to tend to replace it in biological systems, thus bringing about a "conditioned" deficiency of the metabolite. Antimetabolites are of considerably more than academic interest to the practitioner of medicine.

There are naturally occurring antimetabolites, as well as those which are the products of the laboratory. These substances provide the basis for the modern chemotherapy of infectious diseases (i.e., the sulfonamides and the antibiotics), the most effective therapy for many infections ever known to mankind. Through their use we are learning more and more about the metabolic requirements of normal and diseased tissues. As a group they constitute one of the best tools available for the study of the metabolism of malignant growths, and indeed, have been put to practical use in the treatment of some malignancies, particularly the leukemias, with some benefit. These compounds have made it possible to demonstrate that man requires both vitamin B_6 and pantothenic acid—an advance which in the instance of vitamin B_6 led very quickly to the identification of a wide-spread convulsive disorder in infants who had been maintained on a certain proprietary baby food, as due to vitamin B_6 deficiency, and to the discovery that the polyneuropathy occurring in patients with tuberculosis receiving large doses of isoniazid could be prevented by the simultaneous administration of vitamin B_6. The syndrome of vitamin B_6 deficiency in man now is fairly well known and that of pantothenic acid deficiency is being clarified.

Rapid progress is being made in the study and use of the antimetabolites. It seems inevitable that they will become increasingly more utilized in the practice of medicine, not only for the treatment of infectious dis-

* Held in conjunction with the Tenth Annual Meeting of the Members of The National Vitamin Foundation, in New York City, March 1, 1955.

eases and infestations, but also for the study, control, and correction of metabolic aberrations.

For the most effective therapeutic use of the antimetabolites, the physician must comprehend the metabolic requirements of his patient, the metabolic requirements of the infecting organism or abnormal tissue, and the modes of action of the various structural analogues. One of the purposes of the symposium on antimetabolites, held in New York City on March 1, 1955, was to stimulate interest and research in medical applications of accumulated knowledge in some of the less generally well known aspects of this field. Another purpose was to provide the practitioner of medicine and nutrition with information on a most important and rapidly growing branch of medicine which, otherwise, might not be readily accessible to him. It is hoped that publication of the proceedings of the symposium in this issue of the JOURNAL will further advance these purposes.

We take this occasion to thank the speakers for their contributions, which assured the success of the symposium, and we express our gratitude to Dr. Paul L. Day and Dr. Robert W. Heinle, the very able co-chairmen of the symposium.

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Thiamine Antagonists

By LEOPOLD R. CERECEDO, PH.D.

THIAMINE, in the form of the pyrophosphate, plays an important role as a coenzyme in the breakdown of pyruvic acid in living systems. This breakdown may be (a) nonoxidative, as in the case of the degradation of pyruvic acid to acetaldehyde and carbon dioxide, in yeast fermentation; or (b) oxidative, leading to acetic acid and derivatives of this acid.

Since pyruvic acid is a key substance in the intermediary metabolism of carbohydrates, fats, and proteins, it can be expected that a lack of thiamine, or inhibition of the function of this vitamin in the tissues, will lead to grave consequences.

Inspection of the formula of thiamine (Fig. 1) shows that it consists of a pyrimidine ring linked by means of a methylene ($-CH_2-$) bridge to a thiazole ring. Attached to the pyrimidine moiety are two groups, an amino group in position 4, and a methyl group in position 2, which are replaceable by other groups. In the thiazole moiety, there are also two groupings that may be replaced by other groups. They are the hydroxyethyl group in position 5, and the methyl group in position 4. Finally, the thiazole moiety itself may be replaced by a structure containing a pyridine ring. This is not a far-fetched idea, as thiazole compounds show certain chemical reactions also shown by pyridine derivatives.

REPLACEMENT OF THE AMINO GROUP IN THE PYRIMIDINE MOIETY

If we replace the amino group in position 4 of the pyrimidine moiety by a hydroxyl group, we obtain oxythiamine (Fig. 1). This substance was first prepared by Bergel and Todd,[1] who reported that it did not show antineuritic properties in rats. It was also prepared in our laboratory, and was found to be toxic to mice maintained on suboptimal levels of thiamine.[2]

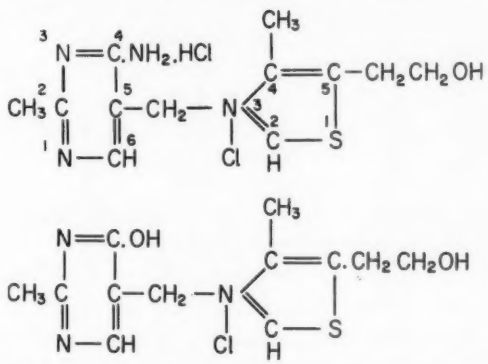

Fig. 1. Structures of thiamine and of oxythiamine.

Jones *et al.*[3] used oxythiamine as a means of producing a controlled deficiency state in mice. That it acts as a thiamine antagonist in the chick has been shown by Daniel and Norris.[4] Frohman and Day[5] found that oxythiamine produced a rise in the pyruvic and lactic acid concentration in the blood of rats. Thiamine counteracted this effect. They also observed that thiamine excretion increased following the injection of the antivitamin. It was found in our laboratory[6] that oxythiamine when administered to mice at a molar ratio of oxythiamine to thiamine of 25:1 or 50:1 had a marked detrimental effect on weight and appetite. Surprisingly enough, the animals did not develop the typical polyneuritic symptoms, but they died in 13–21 days. The competitive nature of the antagonistic action of oxythiamine was clearly shown in a subsequent study.[7] The data, as shown in Figure 2, reveal that, if the effective molar ratio of 25:1 is diminished either by decreasing

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the dose of oxythiamine in the presence of 1 μg of thiamine, or by increasing the dose of thiamine to 2 μg in the presence of 25 μg of oxythiamine, the antagonistic effect disappears.

Bromooxythiamine could be administered at very high levels, ranging from a molar ratio of 50:1 up to 500:1, without showing any toxic effects. The experimental period was 5 weeks, the animals receiving daily in-

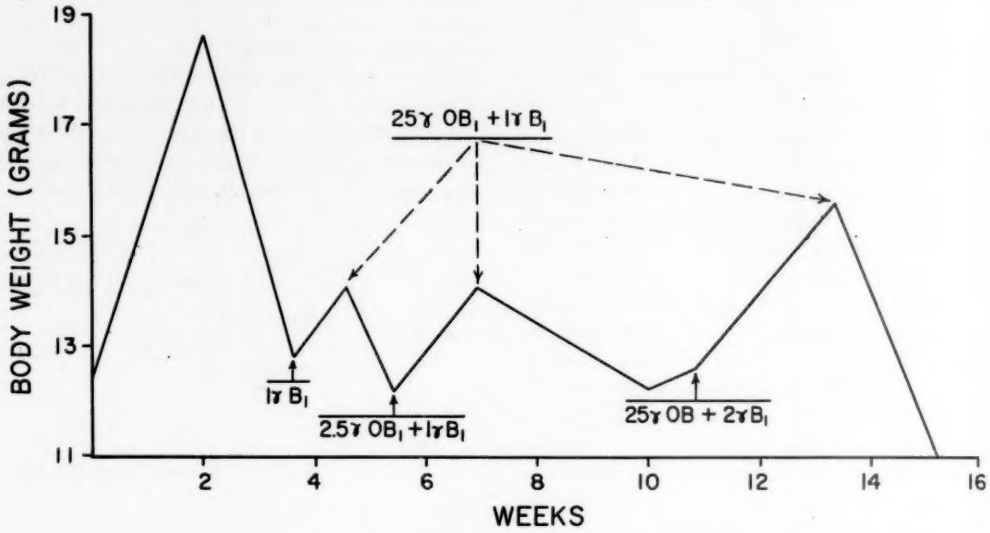


Fig. 2. Growth curve showing the antagonism between oxythiamine and thiamine in mice. From L. R. Cerecedo, M. Soodak, and A. J. Eusebi, *J. Biol. Chem.* 189: 293, 1951.

Replacement of the Methyl Group in Position 2 of the Pyrimidine Moiety

If the methyl group is replaced by an ethyl group, 2-ethyl thiamine is obtained. This derivative possessed full thiamine activity when tested with rats.[8] The corresponding 2-propyl derivative was found to be active in pigeons.[9] On the other hand, the 2-n-butyl derivative (Fig. 3) was found by Emerson and Southwick[10] to produce thiamine deficiency in rats. The effect of 40 moles of the antagonist was counteracted by one mole of thiamine.

Replacement of the Alcoholic Hydroxyl Group in the Thiazole Moiety

With a view of determining in what manner oxythiamine may exert its effects as an antivitamin, we carried out studies with bromo- and chlorooxythiamine.[7] As shown in Figure 4, in these substances the alcoholic hydroxyl group in the thiazole moiety has been replaced by halogen.

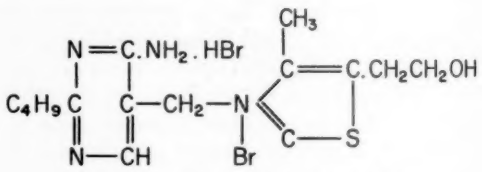


Fig. 3. Structure of 2-n-butyl thiamine.

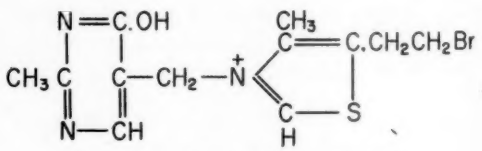


Fig. 4. Structure of bromooxythiamine.

jections of the substance. The results of this experiment are shown in Table I.

Similar results were obtained with chlorooxythiamine. This compound, when given at a molar ratio of 100:1, showed no antithiamine activity.

These findings lead to the following possible explanations for the antagonistic effects of

TABLE I

Effects of Large Doses of Bromooxythiamine in Mice*

Molar ratio	Weight at start of injections	Weight at end of 5th week
	g	g
Controls	11.3–16.4	12.0–17.0
50:1	10.7–12.7	12.0–15.0
500:1	11.2–12.9	12.8–13.8

* From L. R. Cerecedo, M. Soodak, and A. J. Eusebi, *J. Biol. Chem.* 189: 293, 1951.

oxythiamine. It is well known that thiamine is phosphorylated by liver and other tissues of the animal body, and that it is present in organisms to a great extent as the diphosphate (Fig. 5). As far as we know, this is the form

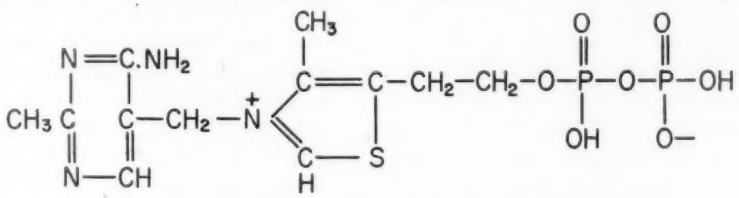


Fig. 5. Structure of thiamine diphosphate (cocarboxylase).

in which it acts as a coenzyme. If it is assumed that oxythiamine is phosphorylated in the animal body in the same manner as thiamine, then the difference in the action of this compound, and that of chloro- and bromo-oxythiamine can be explained. This would indicate that the active form of the inhibitor may be oxythiamine diphosphate. This would combine with the apoenzyme and form an inactive enzyme system. Another possibility would be that oxythiamine may inhibit the phosphorylation of the vitamin, thereby preventing it from becoming functional.

Replacement of the Thiazole Moiety by Pyridine

The pyridine analogue of thiamine was first prepared by Tracy and Elderfield.[11] It was found by Robbins[12] to inhibit the growth of fungi. Woolley and White,[13] who named this antagonist "pyrithiamine," showed it to have potent antithiamine activity in mice.

Subsequently, it was found by Wilson and Harris[14] that the method of Tracy and Elderfield gave a mixture of compounds of unknown structure. They synthesized a new substance which they called "neopyrithiamine" (Fig. 6), and which showed chemical and physical properties that would be expected from the pyridine analogue of thiamine.

The antithiamine effects of oxythiamine and neopyrithiamine in mice were compared in our laboratory.[15] The animals were given a thiamine-deficient diet. When they began losing weight, they were maintained on 1 μg of thiamine per day, injected subcutaneously for one week. Daily injections of oxythiamine and neopyrithiamine were then started, together with 1 μg of thiamine in a molar ratio (antivitamin : thiamine) of 50:1. Both compounds had an immediate effect, causing a drop in the food intake of the mice, and a loss in weight. The data (Table II) show that the antivitamin effect of neopyrithiamine

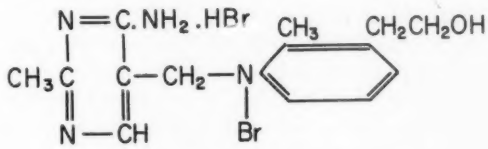


Fig. 6. Structure of neopyrithiamine.

is more pronounced than that of oxythiamine. Additional experiments were carried out in which neopyrithiamine was employed at very

TABLE II

Antithiamine Effect of Oxythiamine and Neopyrithiamine*

Substance Tested	No. of Mice	Wt. of mice at start of injections	Incidence of polyneuritis	Survival time in days
		g	%	
Oxythiamine	9	10.6–13.6	0	13–21
Neopyrithiamine	9	10.2–13.8	100	7–8

* From A. J. Eusebi and L. R. Cerecedo, *Science* 110: 162, 1949.

low levels with a view of determining the molar inhibition ratio.[7] The results obtained are shown in Table III. Judging from the survival time of the animals, the same effect is obtained with neopyrithiamine at a molar ratio of 5:1, as was found at a molar ratio of 50:1. Furthermore, the polyneuritic syndrome observed at the higher levels was also obtained in all cases at the lower levels. Even at a molar ratio of 1:1, neopyrithiamine produced a typical paralysis in all the mice in 13 to 14 days. When the animals reach such a state of deficiency, death usually follows 24 to 48 hours later. It is to be noted that, when administered at the higher levels, neopyrithiamine produces a detrimental effect on the appetite and body weight which is observed overnight. This is not found to be the case when a lower molar ratio of 3:1 or 1:1 is used, with which the mice at first show no harmful effects. They may, in fact, show a slight gain in weight for the first 9 to 10 days. However, on continued injection of the substance, mice begin to lose weight rapidly and develop polyneuritis.

TABLE III

Antithiamine Effect of Neopyrithiamine at Low Molar Ratios*

Molar ratio	Survival time
	days
(Oxythiamine, 50:1)	(13–21)
10:1	7–8
5:1	7–9
1:1	14–16

* From L. R. Cerecedo, M. Soodak, and A. J. Eusebi, *J. Biol. Chem.* 189: 293, 1951, modified.

The above findings on the effects of oxythiamine and neopyrithiamine point to an interesting aspect of thiamine antagonism. *Neopyrithiamine* produces a more acute deficiency, and gives rise to the typical polyneuritic syndrome in the mouse. *Oxythiamine,* on the other hand, does not produce the syndrome; it causes a thiamine deficiency which is similar to that induced by a lack of vitamin in the diet. This difference in the effects of the two antagonists has led to speculations as to its cause.

It has been assumed that neopyrithiamine and oxythiamine attack different systems in the tissues. Woolley and Merrifield[16] find support for such an assumption in their finding that in mice dying of thiamine deficiency caused by neopyrithiamine, the levels of cocarboxylase in the liver, and of pyruvate in the blood were normal. On the other hand, in mice that had been given oxythiamine the levels of blood pyruvate were raised, while the concentration of thiamine in the tissues was depressed. A similar explanation is given by Naber *et al.*,[17] to account for the effects of neopyrithiamine and oxythiamine in the chick embryo, and in young chicks. These workers found that both antagonists inhibited normal embryonic development. Chicks hatching from eggs that had been treated with neopyrithiamine developed polyneuritis. On the other hand, oxythiamine failed to produce polyneuritis. In the young chick, the growth inhibition ratio was 4:1 for neopyrithiamine/thiamine, and 200:1 for oxythiamine/thiamine. As in the embryo experiments, neopyrithiamine gave rise to polyneuritis, whereas oxythiamine caused only local and general tissue edema. Thus, in this species too, the two antagonists exhibit different degrees of toxicity. However, the observations of De Caro *et al.*[18] indicate that more work remains to be done on this problem. They found that administration of neopyrithiamine to rats caused a drop in the total thiamine content in the muscle, liver, and brain.

Effects of Thiamine Antagonists in vitro

Buchman *et al.*[19] studied the effects of the thiazole fragment of thiamine diphosphate ("thiazole pyrophosphate"), and the nonphosphorylated thiazole moiety of thiamine, on the enzymatic decarboxylation of pyruvate by brewers' yeast. The pyrophosphate was found to have an inhibitory effect, whereas the thiazole had none. This difference was explained as being due to a competition between thiamine diphosphate and thiazole pyrophosphate for the specific carboxylase protein. This was an important finding because, according to these workers, it showed "a not hitherto recognized type of competitive inhibi-

tion of enzyme reactions, caused by competition not between substrate and inhibitor, but between coenzyme and inhibitor." These findings have been confirmed in our laboratory.[20,21] In line with these observations are the results of Velluz and Herbain,[22] who have brought about inhibition of the enzyme with oxythiamine triphosphate.

Woolley[23] observed inhibition of yeast carboxylase with neopyrithiamine pyrophosphate, and decreased formation of thiamine diphosphate from thiamine in chicken blood due to neopyrithiamine. However, the amount of antivitamin required in the latter system was very large compared to that which produces thiamine deficiency in the intact animal.

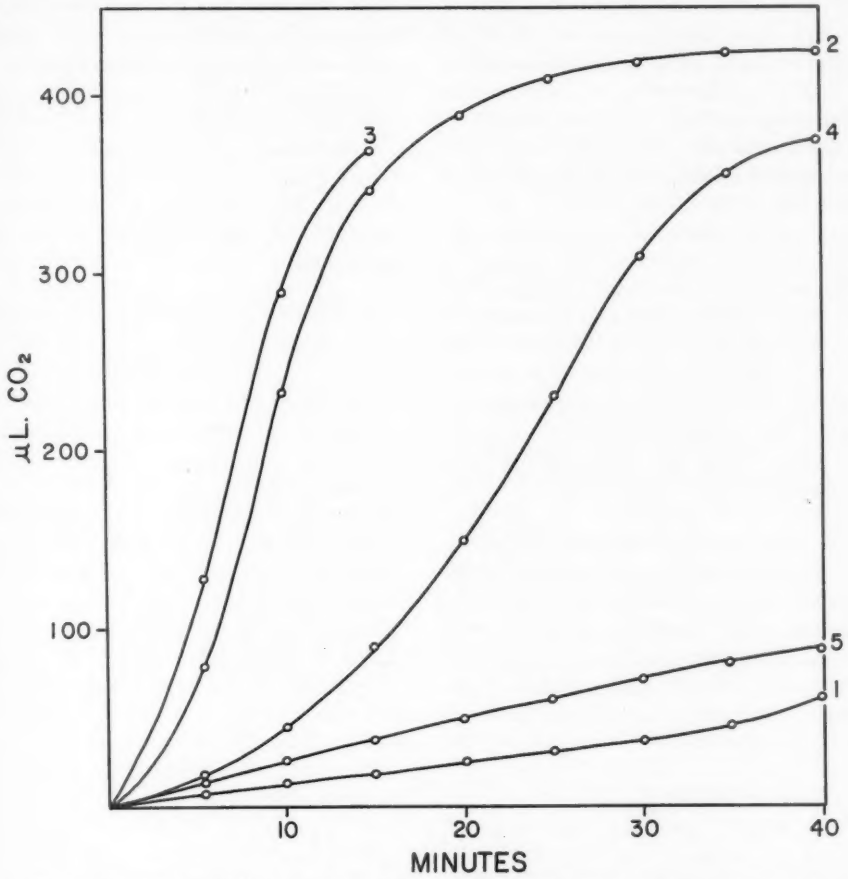


Fig. 7. Effects of oxythiamine and oxythiamine diphosphate on carboxylase activity. Curve 1, reaction mixture, no additions; Curve 2, 7.8×10^{-3} μM of thiamine diphosphate added with reaction mixture; Curve 3, 7.8×10^{-3} μM of thiamine diphosphate with reaction mixture, and 3.88×10^{-1} μM of oxythiamine diphosphate with substrate; Curve 4, 3.88×10^{-1} μM of oxythiamine with reaction mixture, and 7.8×10^{-3} μM of thiamine diphosphate with substrate; Curve 5, 3.88×10^{-1} μM of oxythiamine diphosphate with reaction mixture, and 7.8×10^{-3} μM of thiamine diphosphate with substrate. From S. Eich and L. R. Cerecedo, *J. Biol. Chem.* 207: 295, 1954, modified.

Effects of Thiamine Antagonists on Purified Enzyme Systems

With the publication of methods of obtaining more satisfactory preparations of α-carboxylase by Singer and Pensky,[24] and of thiamine ATP-phosphokinase by Leuthardt and

Nielsen,[25] we have been able to investigate further the mode of action of several thiamine analogues.[26] The effects of oxythiamine, oxythiamine diphosphate, and neopyrithiamine, on the decarboxylation of pyruvic acid by the purified carboxylase are shown in Figure 7.

The data show clearly that the effects of cocarboxylase and oxythiamine diphosphate on the enzyme are determined in large part by the order of addition of the two substances to the reaction mixture. The compound added first was placed in the main compartment of the Warburg vessel in contact with the enzyme and the remainder of the reaction mixture; the second compound was tipped in with the substrate after temperature equilibration. This allowed for approximately 20 minutes between additions of the two to the apoenzyme. Curve 3 shows the lack of inhibition of the system by oxythiamine diphosphate when the analogue is added to the system after cocarboxylase, thus indicating a strong attachment of the coenzyme to the apoenzyme.

On the other hand, introduction of oxythiamine diphosphate before the addition of cocarboxylase results in inhibition (Curve 5). The relative ineffectiveness of oxythiamine as an inhibitor is clearly seen on comparison of Curves 4 and 5. Oxythiamine, added at the same molar levels and in the same manner as oxythiamine diphosphate, has no effect on the carboxylase system. Similar results were obtained with neopyrithiamine.

In an effort to determine whether neopyrithiamine would act in the same manner as oxythiamine diphosphate, if phosphorylated, a small amount was treated according to the method employed by Viscontini *et al.*[27] for the preparation of thiamine triphosphate. An extremely hygroscopic crystalline material was obtained. No attempt at analysis was made, although paper chromatography indicated the neopyrithiamine to have been phosphorylated. This material inhibited the carboxylase system in the same manner as oxythiamine diphosphate.

TABLE IV

Effects of Oxythiamine and Neopyrithiamine on Rat Liver ATP-Thiamine Phosphokinase*

Experiment	Additions	Thiamine diphosphate synthesized per tube
		μg
I	10 μg Thiamine	9.2
	62.5 μg Neopyrithiamine; 10 μg Thiamine after 15 min	1.9
II	10 μg Thiamine	5.4
	1000 μg Oxythiamine; 10 μg Thiamine after 15 min	4.9
	5000 μg Oxythiamine; 10 μg Thiamine after 15 min	3.5

* From S. Eich and L. R Cerecedo, *J. Biol. Chem.* 207: 295, 1954.

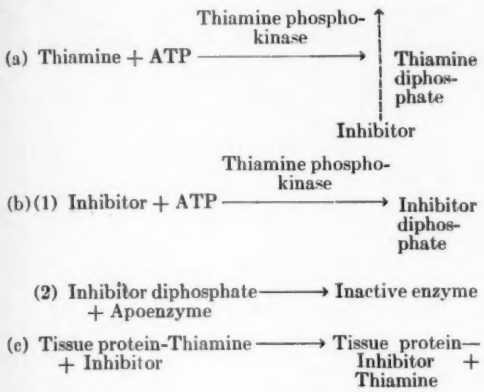
Effects of Oxythiamine and Neopyrithiamine on Rat Liver Thiamine Phosphokinase

Our studies[26] on the rat liver thiamine phosphokinase, which catalyzes the phosphorylation of thiamine in the presence of ATP, revealed an interesting difference between the effects of oxythiamine and neopyrithiamine on the enzyme. These results are shown in Table IV. Whereas the presence of neopyrithiamine at a molar ratio to thiamine of only 5:1 results in a significant inhibition, oxythiamine at levels 100 times those of thiamine is found to be ineffective, and only partially effective at a level of 500:1. The inhibition due to neopyrithiamine can be overcome by the addition of larger amounts of thiamine, indicating the mode of action to be competitive. In contrast to the inhibition involving carboxylase, however, the order of addition of substrate and inhibitor in this case is found to be immaterial.

As a result of these findings, we have been led to the following considerations in an attempt to clarify the mode of action of these antagonists. Since no enzyme system requiring thiamine diphosphate has as yet been found which is inhibited by either neopyrithiamine or oxythiamine, it would seem unlikely that the action of these two compounds is directly upon such a system. We may therefore consider the following three possibilities: (a) The inhibitor may block the synthesis of thiamine diphosphate from thiamine; (b) the

inhibitor may itself be phosphorylated, and the resulting compound could in turn compete with thiamine diphosphate for the apoenzyme; and (c) the inhibitor may in some manner cause thiamine displacement from the tissues.

These three possibilities may be represented as follows:



The experimental evidence regarding two of these possibilities is as follows. With regard to mechanism (a), inhibition of the phosphorylation of thiamine, as stated above, has been definitely shown to be the case with neopyrithiamine, at least with the enzyme system studied. On the other hand, there is no action of oxythiaminc upon the rat liver phosphokinase except at extremely high levels. Such a difference between the two antagonists could possibly explain the aforementioned differences found *in vivo* with mice, and those observed with micro-organisms.[28] Of interest in this connection are certain observations made in our laboratory.[21] It was found that oxythiamine inhibited the formation of thiamine diphosphate from thiamine in a purified yeast preparation. Similar results were obtained with *Propionibacterium pentosaceum*. In contrast, neopyrithiamine did not inhibit phosphorylation in the yeast system.[29] Thus, the results with the rat liver preparation are different from those with the yeast system.

The lack of effect of oxythiamine on the liver phosphokinase is difficult to explain in view of the effects of chlorooxythiamine and bromooxythiamine *in vivo* which have been discussed above. These compounds were found to have no antithiamine activity. Thus, the need for the hydroxyl group for inhibition in the intact animal is shown, suggesting the possible involvement of the substance in a phosphorylation mechanism.

With regard to mechanism (c), elimination of increased amounts of thiamine in the urine of rats after the administration of oxythiamine has been observed by Frohman and Day,[5] and has also been found in our laboratory.[30] Frohman and Day postulate a displacement of thiamine by oxythiamine from enzymes and related proteins. This hypothesis, although inconsistent with the results obtained with rat liver thiamine phosphokinase, could possibly explain the toxic properties of oxythiamine *in vivo*, if we assume thiamine to be stored in combination with a protein.

Effects of Neopyrithiamine and Oxyneopyrithiamine in vivo and in vitro

The different effects of neopyrithiamine and oxythiamine on the rat liver thiamine phosphokinase suggested that the presence of an amino group in position 4 of the pyrimidine moiety might be necessary for inhibition of the enzyme. With this in view, we prepared the 4-hydroxy derivative of neopyrithiamine, oxyneopyrithiamine, and compared its effects with those of neopyrithiamine on the rat liver phosphorylase system, as well as *in vivo*, in the mouse.[31] The results obtained with the enzyme system are shown in Table V. We found that the phosphorylation of thiamine by

TABLE V

Effects of Neopyrithiamine and Oxyneopyrithiamine on Rat Liver ATP-Thiamine Phosphokinase*

The complete system contained 0.5 ml of enzyme preparation, 0.1 ml of 1 *M* $MgCl_2$, 3 mg of ATP, 2.0 ml of 0.1 *M* phosphate, pH 7.4, and 10 μg of thiamine. Total volume 3.4 ml. Incubated 3 hours at 38°C. One ml removed, after boiling, for estimation of thiamine diphosphate.

Additions	Thiamine diphosphate formed
	μg
None (control)	2.5
500 μg Oxyneopyrithiamine	2.6
625 μg Neopyrithiamine	0.2

* From L. R. Cerecedo and S. Eich, *J. Biol. Chem.* (in press).

the rat liver preparation was not inhibited by oxyneopyrithiamine. Neopyrithiamine, on the other hand, when added at the same molar levels as oxyneopyrithiamine, caused an almost complete inhibition of the system.

The results obtained *in vivo* were in harmony with the *in vitro* findings. The mice were placed on a thiamine-deficient diet. The animals began to lose weight after approximately 11 days on the diet. When they were eating one gram of food or less per day, they were injected subcutaneously with 2 μg of thiamine per day, for one week. At this point, the injections of the thiamine analogues were begun. The results of this experiment are shown in Figure 8. The antagonistic effect of neopyrithiamine is shown in animals that received the antivitamin at a molar ratio of 5:1 (Curve B), whereas those that were given oxyneopyrithiamine at the same molar ratio showed a growth rate (Curve C) similar to that of the control group (Curve A).

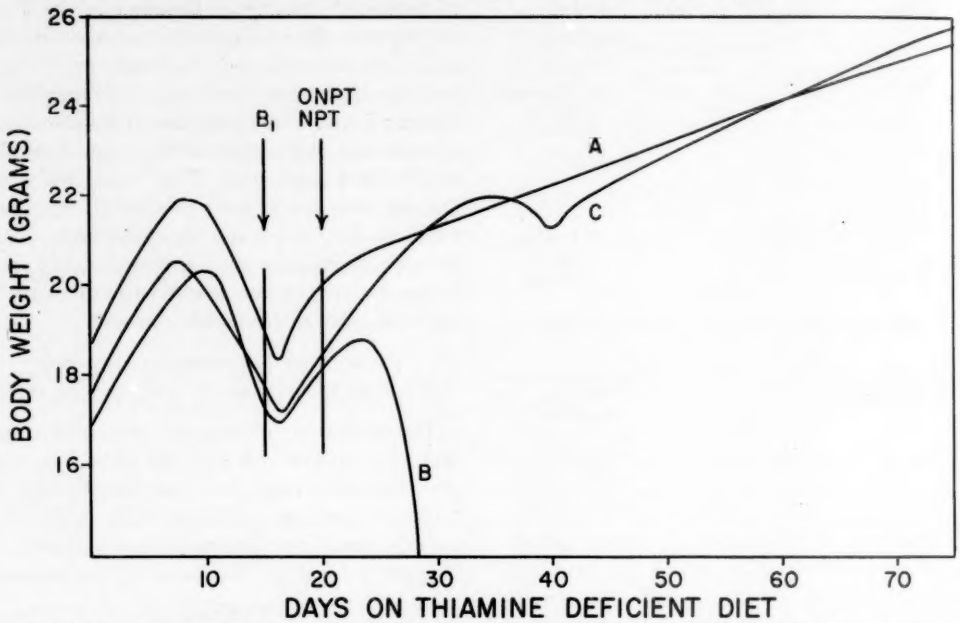


Fig. 8. Growth curves of mice showing effects of neopyrithiamine and oxyneopyrithiamine. Curve *A*, mean body weight of four animals receiving 2 μg of thiamine per day; Curve *B*, four animals receiving 2 μg of thiamine plus 13.5 μg of neopyrithiamine at a molar ratio, antagonist to thiamine, of 5:1. Curve *C*, mean body weight of eight animals receiving 2 μg of thiamine plus 50 μg of oxyneopyrithiamine, 25:1 ratio. From L. R. Cerecedo and S. Eich, *J. Biol. Chem.*, in press.

The above findings indicate that the antagonistic effect of neopyrithiamine depends to a large degree upon the presence of the amino group in position 4 of the pyrimidine moiety. If this group is replaced by a hydroxyl group, a substance is obtained which does not inhibit the enzymatic phosphorylation of thiamine *in vitro*, and does not produce polyneuritis in the intact animal.

It is of interest to compare the antithiamine effects of oxythiamine and neopyrithiamine. Both compounds contain a hydroxyl group in position 4 of the pyrimidine moiety. They differ in that the thiazole ring of oxythiamine has been replaced by a pyridine ring in oxyneopyrithiamine. Neither substance will inhibit the phosphorylation of thiamine by the liver phosphokinase, nor do they cause the typical polyneuritic syndrome in mice. However, as was shown above, oxythiamine is an effective thiamine antagonist *in vivo*. For example, at a 25:1 molar ratio, it will cause an antithiamine effect in mice, while no such effect is obtained with oxyneopyrithiamine at

this level, or even at a 50:1 ratio. Thus, the substitution of the thiazole for the pyridine ring in oxythiamine results in the disappearance of the antagonistic action. It would seem that oxythiamine is sufficiently related to thiamine to function as an antagonist, while oxyneopyrithiamine, further removed structurally from the vitamin, is now outside the range of antagonistic effectiveness.

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Riboflavin Antagonists

By JOHN P. LAMBOOY, PH.D.

THE RECENT appearance of several articles[1-4] dealing with the biological activity of compounds structurally related to riboflavin makes it unnecessary to dwell on this subject in detail at this time. I would like to introduce a few theoretical considerations concerning the structure of such compounds which will serve both to limit severely the number of compounds to be discussed and to simplify the discussion of the mechanism by which these compounds produce enzymatic inhibition.

Most of the observations on which these considerations are based were made by R. Kuhn and his associates and are related to the sites on the riboflavin molecule which link with the protein of enzymes[5,6] and to the variations in the redox potential of the compounds which result from modifications of the molecule.[7] Using the "old yellow enzyme" as a detector, they found that riboflavin-5′-phosphate (RP) is linked to the protein by means of the phosphate, and the imino group in the 3 position. Of these two attachment sites, the 3-imino group is the most critical, since riboflavin will link to an appreciable extent without a 5′-phosphate group, while even the presence of a 5′-phosphate will not afford linkage if the hydrogen in position 3 has been replaced by a methyl group. If these two attachment sites are available, very extensive alterations may be made at positions 6 and 7 without destroying the ability of the flavin to become attached to the protein.

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MOLECULAR CONSIDERATIONS

In regard to the ability of flavins to link to protein, it appears that from the standpoint of structure the most specific characteristics are located in that portion of the molecule which extends from the 5′-phosphate to the 3-imino group. It is not surprising to find, therefore, that only limited alterations can be tolerated in the polyhydric alcohol chain at position 9 without some sacrifice in the ability of the flavin to link to protein. This does not imply that these characteristics are essential for linkage, since there are two points of attachment, as described above. While either point may accomplish some linkage, it seems reasonable that if both points can become attached to protein, the association will be more readily accomplished or more firmly maintained. The likelihood of making such a two-point attachment appears to be greater as the structure of the flavin more nearly resembles that of riboflavin.

It has been demonstrated that L-araboflavin can link with enzymes,[5,6,8] and it is interesting to note that flavin-type compounds known to date which have appreciable activities either as vitamins or inhibitors (linkage to protein apparently being required in both cases) have a common configuration about the 2′-position. This point is illustrated by the structural formulae shown in Figure 1. Kuhn observed that the configuration about this position was one of the points of similarity between riboflavin and L-araboflavin, and suggested that it might be of importance in the activity of the latter.[5]

These portions of the molecule which are so critically involved in protein association may be altered extensively with relatively little influence on the redox potential of the compound. We find, for example, that the

polyhydric alcohol chain may be D-ribityl, D-xylityl, or D-dulcityl, with only small changes in the E_0' values; conversion from ribityl to methyl also involves only a moderate change in E_0'. Methylation of the nitrogen in the 3 position also has only moderate influence on the redox potential of the compound.

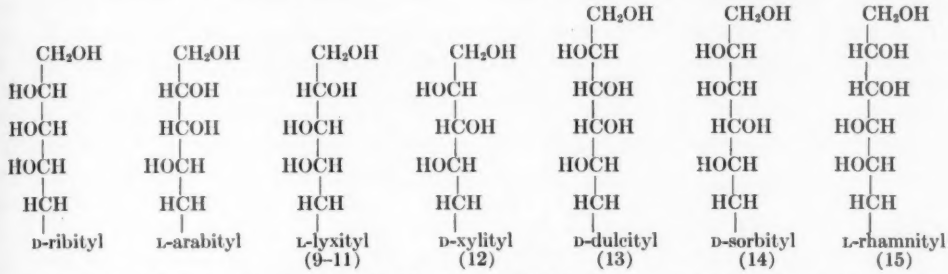


Fig. 1. Structural formulae of various polyhydric alcohols which have been combined with flavins to form compounds with vitamin or inhibitor activity.

The structures of the substituents in positions 6 and 7, while having little influence on the flavin's ability to link with the enzyme, do have a pronounced influence on the oxidation-reduction potential of the compound. If the methyl groups are replaced by chlorine atoms, the E_0' value is made 0.09 volt more positive. Even greater changes in E_0' values result from changes in position of the substituent groups. If the methyl group in position 7 of lumiflavin is moved to the 8 position, the E_0' value of the new compound is 0.113 volt more positive than that of lumiflavin.

In general, in the synthesis of homologues and analogues of riboflavin, it appears to be desirable to maintain the 9-(1′-D-ribityl)-isoalloxazine configuration in order to favor linkage between the flavin and enzyme protein. In attempting to produce riboflavin inhibitors, we have done this while at the same time varying the nature of the substituents in the 6 and 7 positions, with the purpose of altering the redox potential. This approach has an additional appeal in that, if the redox potential is not altered excessively, any compound obtained which was not an inhibitor may turn out to have vitamin properties for some organisms. At the outset, it seemed reasonable to assume that if the change in potential were very small, the compound might have vitamin activity even for organisms whose metabolic oxidation-reduction reactions are narrowly limited. Greater changes would diminish the possibilities of obtaining vitamin activity, while increasing the chances of finding inhibitory activity, until ultimately a sufficiently large change might render the compound devoid of biological activity.

New Compounds

The first of the current group of riboflavin inhibitors was 6,7-dichloro-9-(1′-D-ribityl)-isoalloxazine, or dichloroflavin.[16] When added to cultures of *Staphylococcus aureus* or *Streptobacterium plantarum*, neither of which require an external source of riboflavin, these compounds were potent inhibitors during the early growth periods. However, as the growth periods were lengthened, the ratios of the quantity of inhibitor to riboflavin required for suppression of the growth of the organisms became increasingly greater. (These ratios are expressed in terms of the inhibition index, that is, the quotient resulting from the division of the number of moles of inhibitor by the number of moles of the vitamin in question.) This phenomenon constitutes a reversal of the inhibition observed with short growth periods, and it may have been due in part to the synthesis of riboflavin by the organisms and in part to the development of some degree of adaptation of the organisms to the inhibitor. When tested on *Lactobacillus casei*[11] in the presence of riboflavin, dichloroflavin was found to be completely inert.

Soon after this work was published, 6,7-dimethyl-9-(1′-D-dulcityl)-isoalloxazine, galactoflavin,[13] was reported as an inhibitor of riboflavin in the rat. The available data do

not permit the determination of a numerical value of the index for 50 per cent inhibition, but since the value seems to lie between 40 and 60, the compound can be considered a good inhibitor in the case of the rat. When tested on *L. casei*,[11] the compound was found to stimulate growth at low levels in the presence of small amounts of riboflavin, but to inhibit at high levels. The inhibition index for 50 per cent of normal growth is about 1000. 5,6-Dimethyl-9-(1′-D-ribityl)-isoalloxazine, isoriboflavin, produces the same result[11] in *L. casei*, with an inhibition index of about 40.

Diethyl Riboflavin

We have synthesized four new flavins related to riboflavin by following the general plan outlined above, and have found them all to possess interesting biological activities. The first of these, 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine, diethyl riboflavin,[1,17] was found to replace riboflavin completely for *L. casei*[18] and *Bacillus lactis acidi*.[19] Riboflavin-deficient rats receiving this material showed responses ranging from weight maintenance with increased survival time to exceptional weight gains with reduced survival time, depending on the quantity of material administered.[19] At very high levels, this compound is a potent competitive[20] inhibitor of riboflavin, with an inhibition index for 50 per cent reversal of 6.

6-Ethyl Riboflavin

6-Ethyl-9-(1′-D-ribityl)-isoalloxazine, 6-ethyl riboflavin,[21] has about 3 per cent of the activity of riboflavin for *L. casei* and shows no inhibitory properties for this organism when present at 9000 times the level of the accompanying riboflavin.

6-Chloro-7-methyl Riboflavin

6-Chloro-7-methyl-9-(1′-D-ribityl)-isoalloxazine, 6-chloro-7-methyl riboflavin,[22] is an extremely interesting compound. When this compound is added to cultures of *L. casei* at the level of 2.5 μg per ml in the presence of 0.03 μg per ml of riboflavin, the growth rate is reduced to 50 per cent of the normal value. The inhibition index is 80. When the analogue is given to groups of riboflavin-deficient rats[23] at levels of 6, 12, or 24 μg per day, the growth response is very nearly identical with that produced in other deficient groups receiving 3, 6 or 12 μg per day, respectively, of riboflavin. At 50 μg per day, animals grow at the same rate on the analogue as they do on 50 μg per day of riboflavin, but 60 per cent of the experimental animals die. Groups of normal animals receiving 500 μg and 2 mg per day all die by the eighth and the fourth day, respectively. The growth rate is the same for groups of deficient animals receiving 50 μg of analogue, 500 μg of analogue plus 40 μg of riboflavin, or 500 μg of analogue plus 200 μg of riboflavin per day, but 60 per cent of the first group die, all of the second group die, and all of the last survive. The derivation of an inhibition index is difficult under these circumstances.

7-Chloro-6-methyl Riboflavin

When the chloro- and methyl groups are reversed to form 7-chloro-6-methyl-9-(1′-D-ribityl)-isoalloxazine, 7-chloro-6-methyl riboflavin,[22] a remarkable change in biological activity occurs. The growth of *L. casei* is reduced to 50 per cent of normal when the levels of analogue and riboflavin are 0.7 μg per ml and 0.03 μg per ml, respectively; the inhibition index is 22. This compound, which is so much more potent than its isomer in the inhibition of *L. casei*, is almost completely devoid of biological activity in the riboflavin-deficient rat.[24] At levels of 25 to 50 μg per day, the animals fare no better than the deficient controls. At 500 μg to 1 mg per day, they show a moderate gain and better survival than the former groups. When given 500 μg of analogue plus 20 μg of riboflavin per day, they grow somewhat less than those receiving 20 μg per day of riboflavin.

Mechanisms

In considering possible mechanisms for the action of riboflavin analogues, it is worth while to summarize the general types of metabolic changes which riboflavin can undergo. These may be illustrated as shown in Figure 2.

It is known that a rat may die of riboflavin deficiency and still retain relatively large amounts of the vitamin in its tissues. This suggests either that life is compatible only with high tissue levels of the vitamin, or that the holoenzymes are not readily dissociated to liberate the coenzymes for redistribution among the enzymes whose biochemical insufficiency results in cessation of growth and eventual death. Both possibilities may be important, but for the moment I would like to emphasize the second.

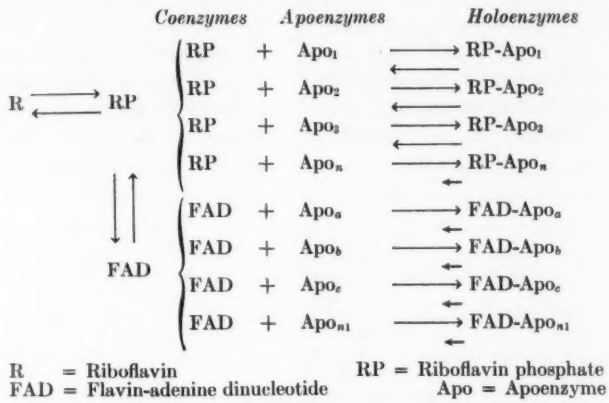


Fig. 2. Scheme of types of metabolic reactions of riboflavin.

Bessey and associates[25] have shown that the total riboflavin in the liver of the rat is distributed as riboflavin (R), riboflavin phosphate (RP), and flavin-adenine dinucleotide (FAD) to the extent of 2, 16 and 82 per cent, respectively. If the hypothesis is correct that flavoproteins are not readily dissociated, it looks very much as though the FAD-containing flavoproteins must immobilize most of the riboflavin.

When riboflavin-deficient rats are given suitable levels of diethyl riboflavin, they resume growth and recover from the outward signs of the deficiency; the rate and extent of growth depend on the level of the flavin administered. If the level is low, they grow only a little, but survive longer than deficient controls. If the level is high, they grow considerably, but die before the deficient controls. At the time these observations were reported,[19] we proposed that the observed growth was due to the displacement, by diethyl riboflavin, of riboflavin in some form from the tissues.

There appear to be two possible ways by which riboflavin might be released from the tissues, and the process depends upon the functional state of the riboflavin which is present. If, on the one hand, all of the flavoproteins have become uniformly and simultaneously depleted of their coenzymes, it may be considered that they all become limiting factors toward growth. If this is the case, it follows that growth resulting from the administration of diethyl riboflavin must be due to the ability of diethyl riboflavin derivatives, produced in the body from diethyl riboflavin, to function biochemically when combined with those apoenzymes from which the normally occurring riboflavin derivatives have been displaced. Those displaced normal riboflavin derivatives are now free to associate with other apoenzymes from which riboflavin cannot be displaced, or with which diethyl riboflavin derivatives cannot form biochemically functional complexes. If, on the other hand, the process of depletion of coenzymes has not been uniform and simultaneous, then we may assume that only one, two, or a few reactions are limiting. The other holoenzymes must then be more fully saturated with normal riboflavin derivatives than they need be to carry out the enzymatic functions demanded of them under these abnormal con-

ditions. This surplus of riboflavin derivatives may be viewed as a pool of biochemically unavailable riboflavin. If this is the case, one could account for the growth-stimulating properties of diethyl riboflavin without requiring it to possess activity as a coenzyme. The diethyl riboflavin in the form of some derivative would then displace riboflavin derivatives from this otherwise unavailable pool. It appears that one or the other of these mechanisms must be valid, or else we must assume that some flavoproteins are uniquely required for growth and some others for survival.

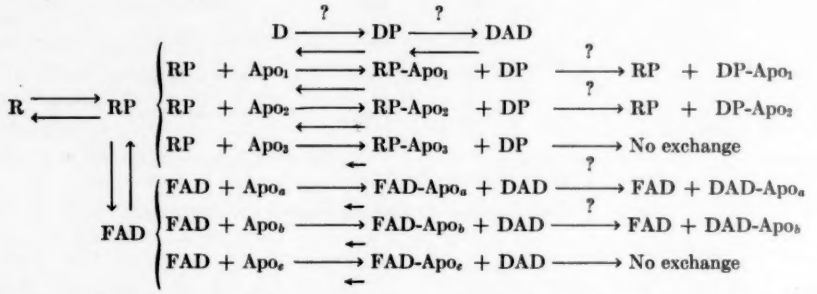

D ? DP ? DAD
R RP
RP + Apo1 RP-Apo1 + DP ? RP + DP-Apo1
RP + Apo2 RP-Apo2 + DP ? RP + DP-Apo2
RP + Apo3 RP-Apo3 + DP No exchange
FAD
FAD + Apoa FAD-Apoa + DAD ? FAD + DAD-Apoa
FAD + Apob FAD-Apob + DAD ? FAD + DAD-Apob
FAD + Apoc FAD-Apoc + DAD No exchange

Fig. 3. Mechanism originally proposed for the displacement of normal riboflavin derivatives from flavoproteins by diethyl riboflavin derivatives.

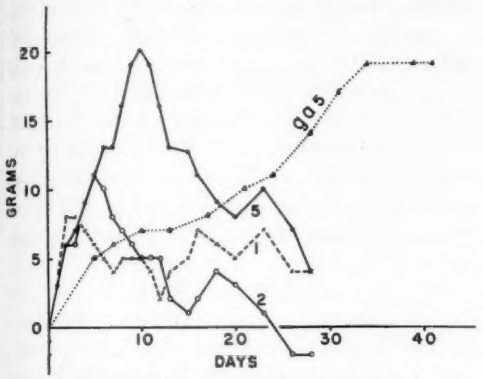
Displacement Hypothesis

The exchange of diethyl riboflavin coenzymes for normal riboflavin coenzymes postulated above could not have produced functional holoenzymes in all cases in the rat, as it must do in *L. casei*, for example, or the animal would have survived. In the past[19] we have ascribed the fatal effects of the high levels of diethyl riboflavin to this process of displacement, plus an actual competition with riboflavin for critical enzyme sites. We referred to the displacement of riboflavin—the term riboflavin was used in a collective sense—since we could not be more specific at that time. The actual form of such displaceable riboflavin must be, of course, riboflavin phosphate (RP) and flavin adenine-dinucleotide (FAD), these two forms constituting 16 per cent and 82 per cent, respectively, of the total riboflavin present in at least the liver of the rat. The details of the mechanism of displacement described above, which we proposed in much less detail on an earlier occasion, are illustrated in Figure 3.

The displacement hypothesis just described was tested indirectly in another way. Normal young rats were placed on a riboflavin-deficient diet, and separate groups were given 2 mg per day of diethyl riboflavin for 1 day, 2 days, and 5 days. The growth shown by these groups during a period of 28 days was very different[26] from that shown by a group maintained on the deficient diet. The treated groups grew at a very rapid rate for approximately twice as many days as they had received the diethyl riboflavin, and then lost the weight gained during the remaining days of the test period. At the end of the 28 days, the treated animals appeared to be in a state of riboflavin deficiency. The untreated controls on the riboflavin-deficient diet gained weight during the entire period, and after 28 days had not reached a state of deficiency (Fig. 4).

Since a displacement mechanism appeared to furnish the best answer to the question of how diethyl riboflavin acts, we turned our attention to the direct demonstration of a displacing agent and of such a displacement mechanism. If we assume that a riboflavin-deficient rat will excrete a relatively constant level of riboflavin in his urine, then the displacement of riboflavin by diethyl riboflavin should be reflected by an elevated excretion of riboflavin. Having available no suitable differential assay procedure for distinguishing between riboflavin and diethyl riboflavin, we

turned to chromatography, but learned nothing about displacement with this approach. When diethyl riboflavin was administered in high levels, its excretion in the urine was much greater than that of riboflavin. The difference in concentration was so great that when



ga 5—Riboflavin deficient (6% gum acacia)
5—2 mg diethyl riboflavin (in 6% gum acacia) for 5 days
2—2 mg diethyl riboflavin (in 6% gum acacia) for 2 days
1—2 mg diethyl riboflavin (in 6% gum acacia) for 1 day

Fig. 4. The growth of normal weanling rats on 2 mg per day of diethyl riboflavin for 1, 2, and 5 days, compared to animals on a flavin-free diet.

the urine was diluted sufficiently to produce a discrete diethyl riboflavin spot, the riboflavin spot was no longer visible. We were unable to measure differences in quantity of riboflavin excreted under these circumstances.

We next tried to discover whether the tissues of the rat could phosphorylate diethyl riboflavin. We failed again this time because we were unable to obtain preparations, which have been reported to phosphorylate riboflavin, in a state free of riboflavin and its coenzymes. Such preparations, therefore, seemed unsuited for the task at hand, since it was impossible, owing to the lack of reference material, to know where to look on our chromatograms for the spots corresponding to diethyl riboflavin derivatives. Thus, the latter spots might have been obscured by spots produced by riboflavin derivatives. In any case we could produce no evidence by our procedures that the preparations were able to phosphorylate riboflavin.

Our next effort was direct. It should be possible to measure total flavin in riboflavin-deficient rats as riboflavin, and in the experimental animals as riboflavin plus diethyl riboflavin. If all the riboflavin and diethyl riboflavin derivatives were converted to the simple flavins, we planned to separate the riboflavin and diethyl riboflavin chromatographically and then determine their quantities independently. Any reduction of riboflavin found in the experimental group would be a measure of that displaced from the apoenzymes. The actual separation and quantification of artificial mixtures of these flavins was perfected, only to discover difficulty from an unexpected source. We tried a number of procedures, but none accomplished the complete hydrolysis of RP and FAD. One disturbing observation was that in procedures which made use of 0.1 *N* hydrochloric acid for hydrolysis with the tissues of normal animals we obtained chromatograms bearing five flavin spots. Three of these were clearly R, RP, and FAD. We do not know the identities of the other two, but they can be produced by treating pure riboflavin solutions by this method.

Recent Studies

In an effort to overcome all of the difficulties mentioned above, we turned to a consideration of the use of radioactive isotopes. If the riboflavin to be displaced were radioactive, we could assay the excreted material by direct counting techniques. Haley* embarked on and accomplished the synthesis of radioactive riboflavin[27] specifically for this purpose, but before we put it to use in this problem, other observations bearing on the study of this displacement were being made.

The first of these developed when Aposhian† turned to the approach which should

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A large part of the work referred to in the text of this article was done by these two men while graduate students in the Dept. of Physiology of the University of Rochester.

have been our first one, and which proved to be fruitful in that it solved a portion of the problem at hand. Using a combination of the procedures described by Crammer[28] and Yagi,[29] Aposhian made warm water extracts of the livers of normal animals. When these extracts were chromatographed, they produced clear-cut spots for R, RP, and FAD. The same treatment, when applied to the livers of animals which had received diethyl riboflavin, produced these three spots and two new spots. One of the latter was promptly identified as diethyl riboflavin, while the Rf value of the other suggested that it might be diethyl riboflavin phosphate. Aposhian then synthesized diethyl riboflavin phosphate, and it proved to be identical with the flavin in the second unknown spot.[30]

The rat is able, therefore, to convert diethyl riboflavin into diethyl riboflavin phosphate, and the latter compound may very well be our postulated displacing agent. We may now revise the mechanism shown in Figure 3 to the following one, shown in Figure 5.

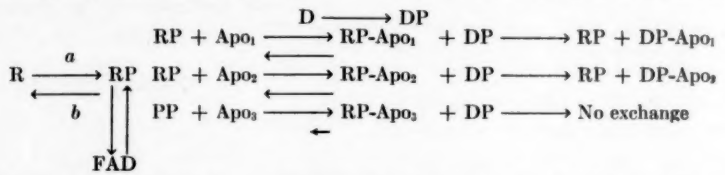

Fig. 5. Currently proposed mechanism for riboflavin displacement by diethyl riboflavin phosphate.

The fatal effects of high levels of diethyl riboflavin may be due to the action of the phosphate of this compound in displacing RP from enzymes with which this phosphate cannot form functional holoenzymes, or to the more conventional postulated type of inhibition of reaction *b* (Fig. 5). That inhibition is caused by competition at reaction *a* seems highly unlikely. It also appears unlikely that diethyl riboflavin phosphate is converted into a derivative corresponding to FAD. The absence of such a derivative from the chromatograms points strongly to reaction *b* as being a limiting one.

Discussion

Diethyl riboflavin appears to function in the following manner: in the form of its phosphate it displaces riboflavin phosphate from a number of apoenzymes with which the latter normally functions as a coenzyme. The new "diethyl-flavoproteins" are biochemically functional. The liberated riboflavin phosphate is now free to link with other apoenzymes which are not functional without it, or are not subject to exchange because of unfavorable dissociation constants; or it may be converted to FAD and function in the usual manner. Competition at point "b" during the administration of large amounts of the homologue must be viewed as a condition developing as a result of rising levels of diethyl riboflavin phosphate and falling levels of riboflavin phosphate.

The fact remains that we have not yet demonstrated that the postulated displacement mechanism is valid. Our newly available radioactive riboflavin apparently could be useful in solving this problem, although in view of the second observation bearing on displacement it may turn out to be unnecessary. The second observation bearing on displacement resulted from Haley's synthesis of, and rat growth studies with 6-chloro-7-methyl riboflavin. This compound appears to give rise to a far more potent displacing agent than does diethyl riboflavin and can cause the displacement of riboflavin from tissues of the rat when administered at extremely low levels. The levels which appear to bring about displacement are so low that the presence of this compound in the tissues cannot interfere with the direct determination of riboflavin by microbiological procedures. By what may prove to be a fortunate coincidence, this is the chloro-methyl riboflavin isomer which is the least toxic for *L. casei*, and as long as the ratio of this inhibitor to riboflavin remains 65 (or less) to one, this organism grows as if only riboflavin were present.

One must admit, however, that it seems a little unlikely that this more potent displacing agent can form biochemically functional "flavoproteins." It may develop that there is a reservoir of bound riboflavin in animal tissues which serves no biochemical function under normal conditions and from which riboflavin can be displaced.

Medical Implications

The medical implications which can be derived from the known riboflavin inhibitors and our very meager knowledge of their mechanism of action are obviously not impressive. For the present, we have some hope that 7-chloro-6-methyl riboflavin, which is a potent inhibitor for some micro-organisms and is apparently relatively harmless to the rat at least, may have some chemotherapeutic possibilities.

We may also draw some encouragement from the fact that a related compound, 6,7-dichloro-9-(1′-D-sorbityl)-isoalloxazine,[14] causes the regression of lymphosarcoma implants in mice, although this may not be due to a specific interference with riboflavin metabolism.

Atabrine, while hardly structurally related to riboflavin, does appear to owe a large measure of its antimalarial activity to the inhibition of certain flavoproteins. The fact that so few pathogens require an extraneous source of riboflavin, while most of their hosts do, suggests that only a riboflavin analogue which possesses unique properties may find chemotherapeutic application. Such unique properties must be sufficiently near to those of riboflavin so that the less critical metabolic requirements of micro-organisms can be tricked by the compound, and yet, these properties must make the compound sufficiently unlike riboflavin so that the more critical metabolic requirements of the host will exclude it altogether.

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Vitamin B_6 Antagonists

By W. W. UMBREIT, PH.D

VITAMIN B_6 is a catalyst of considerable biological importance. Studies during the past 15 years have established a key role for this vitamin in protein and amino acid metabolism and, somewhat less firmly, in fat metabolism. It would seem, therefore, that suitable antagonists to this vitamin might be of therapeutic importance. We are actually in a most curious situation with respect to vitamin B_6 and its antagonists—so that I trust the present discussion is not a mere repetition, for yet another vitamin, of further examples of chemical alteration of the vitamin molecule providing substances antagonistic to the vitamin, some of which may possess clinical value. To possess therapeutic significance, such antagonists must have properties other than mere antagonism.

The properties of the vitamin B_6 group and the nature of the antagonists have been recently and adequately reviewed.[1,2,3,4] I shall, therefore, concentrate my attention upon some newer aspects of the problem of vitamin B_6 antagonists, particularly those having some therapeutic implications.

The field for antagonists in the vitamin B_6 group, with its many related members, would seem to be wide enough. I shall use the term vitamin B_6 to designate all active members, and the specific common names for particular members of the group. As illustrated in Figure 1, at present vitamin B_6 comprises pyridoxine, pyridoxamine, pyridoxal and their respective phosphates. There doubtless exist in nature and certainly have been prepared in the laboratory several substances which, upon breakdown, yield vitamin B_6, as, for example, the pyridoxylidene amines, the pyridoxyl amines,[5] and the pyridoxylamino acids.[6,7]

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THE ACTIVE FORM

It appears that vitamin B_6 exerts its action by being converted into pyridoxal-5-phosphate, which is the prosthetic group of a variety of catalytic proteins. Pyridoxal-5-phosphate is known to be the coenzyme of the amino acid decarboxylases, of transaminases, and is associated with enzymes concerned with the breakdown of tryptophan, and its synthesis, the breakdown of serine, threonine, cysteine, the cleavage of thioethers, and several related reactions.[2] In all of these cases pyridoxal-5-phosphate is the active coenzyme; and, with one exception, pyridoxamine phosphate and pyridoxine phosphate are not active, nor are the unphosphorylated bases. This exception is found in the activity of pyridoxamine phosphate in transaminase.[8,9] There are, in addition, transaminases which transaminate pyridoxamine, and certain purines[10] and enzymes also exist which phosphorylate pyridoxal and certain other pyridoxine derivatives.[11] In fact, so general is the involvement of pyridoxal phosphate with the reactions of the amino acids, that today it is the first coenzyme one looks for in any reaction of amino acids, and it frequently occasions surprise when it is not the coenzyme. The requirement for vitamin B_6 in the rat is dependent upon the protein intake, and under circumstances of vitamin B_6 deficiency various aspects of protein synthesis and degradation are impaired.[1,2,4] Somewhat less precise, but nonetheless real, is the increasing evidence, some confusing, all very general, of the relationships between vitamin B_6 and fatty acid requirements.

There are, of course, many alterations possible in the molecules of vitamin B_6, but since pyridoxal-5-phosphate is the active form it is evident that if unreactive groups are placed at position 4 or 5 the substance cannot act

as the vitamin, and may possibly be an antagonist. By placing a methyl at position 4 one has what is usually called desoxypyridoxine. While modern terminology would call this deoxypyridoxine, we will retain the term desoxypyridoxine. Another desoxypyridoxine is obtained by placing a methyl at position 5, frequently called 5-desoxypyridoxal. There is another antagonist in which the 4 position has been replaced by a methoxy methyl, usually called methoxypyridoxine. These relationships are illustrated in Figure 1.

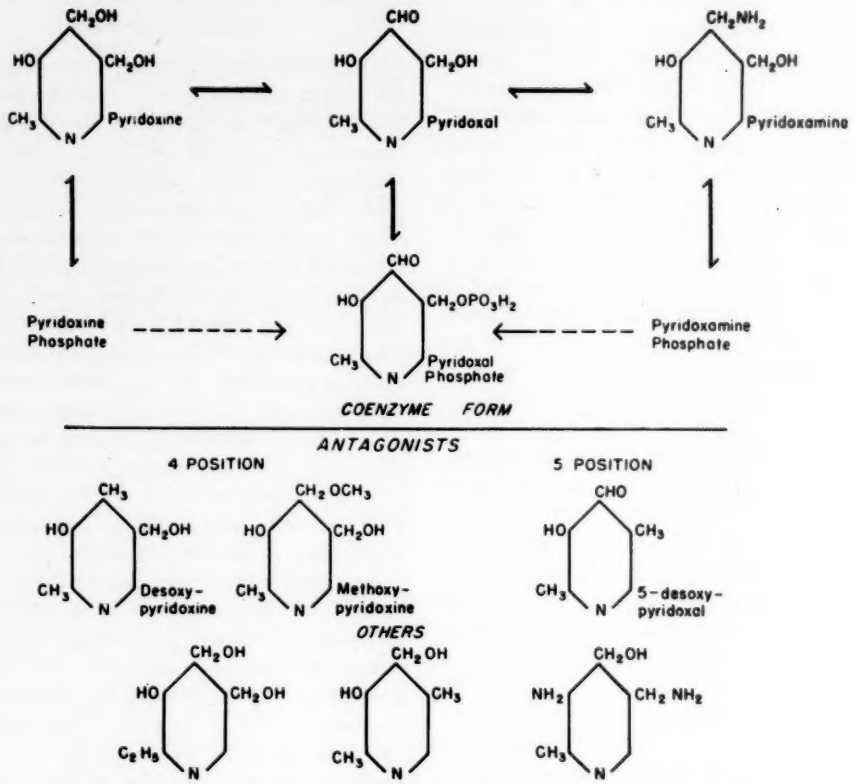

Fig. 1. Relationship of the members of the vitamin B_6 group and their antagonists.

Pyridoxine, pyridoxal, and pyridoxamine are interconvertible, and by way of a reaction with ATP are converted into their respective phosphates. It may have been demonstrated somewhere that the phosphates are interconvertible *per se* by biological systems. Certainly the phosphates are interconvertible chemically, but I do not happen to know of any direct conversion by biological systems. While many alterations in the molecule of vitamin B_6 can be made, the compounds resulting from such alterations are relatively inert, except for those illustrated in the figure. Of these, three have been given trivial names: desoxypyridoxine, methoxypyridoxine, and 5-desoxypyridoxal. These and others interfere with the utilization of vitamin B_6 in one or another organism. The one of greatest importance, or at least the one receiving the most adequate study, is *desoxypyridoxine*, and our attention will be concentrated on it.

Desoxypyridoxine

There is a wide and significant literature on the effects of vitamin B_6 deprivation upon brain excitability,[12,13] adrenal function,[14] reproduction,[15] arteriosclerosis,[16] hypertension,[17]

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disorders of the skin,[18,19] etc. In most of these, particularly in humans,[20–24] the deficiency has been brought about by using not only a vitamin B_6-deficient diet, but desoxypyridoxine as well (the 4-desoxy compound is meant). These experiments generally employ the assumption that the vitamin antagonist merely accentuates the deficiency. The lesions observed are usually rapidly reversible by administration of vitamin B_6, and are prevented from developing by a normal amount of vitamin B_6. Yet there are several indications that, in reality, one is dealing, in 4-desoxypyridoxine antagonism, with phenomena not all attributable to frank vitamin B_6 deficiency.

It is in this respect that the action of desoxypyridoxine is of somewhat more interest than simple vitamin competition and offers some possibilities of therapy—the latter, so far, not too well pursued. The correspondence between the observations on straight deficiency and administration of desoxypyridoxine are not always all they should be, which led Stoerk[25] to describe "acute" (that produced by desoxypyridoxine) compared to "chronic" deficiency, and to point out that the following characters were associated with the acute deficiency—that is, that the following were observable when desoxypyridoxine was present, namely:

1. A dermatitis of the tail in rats.
2. A lack of response to antigen.
3. The occurrence of Tyzzer's disease in mice.
4. Regression of lymphosarcoma implants in mice.

And finally, one which perhaps because of the long list of others, seemed to have been forgotten:

5. No fall in the vitamin B_6 content of the liver (or other organs).

This is particularly important since in this case one has the symptoms of vitamin B_6 deficiency without actual tissue deficiency of the vitamin.

When a rat or mouse is placed on a vitamin B_6-deficient diet, after a period of 3 to 4 weeks it develops certain characteristic symptoms. If the tissues from such an animal are analyzed it will be found that the total vitamin B_6 content has decreased, the pyridoxal phosphate content has decreased,[25,26,27] and, if one measures the activity of the enzymes, transaminase in heart and liver,[27] or glutamic decarboxylase in brain,[28] these too will have decreased. If desoxypyridoxine is added to this vitamin B_6-deficient diet, the symptoms appear much sooner; superficially they seem the same and are completely alleviated by a supply of vitamin B_6. But under such "acute" deficiency, the vitamin B_6 content remains essentially the same as normal,[25,26,27] the pyridoxal phosphate content is high,[25,27] and the activity of transaminases of liver and heart,[27] and the activity of glutamic decarboxylase in the brain[28] remain essentially unaltered, unless the deficiency is allowed to go to really serious lengths. That desoxypyridoxine is antagonized by vitamin B_6 there is no question; but its action involves more than the antagonism of the vitamin. One might almost say that while vitamin B_6 is an antagonist for desoxypyridoxine, it looks as though desoxypyridoxine is not an antagonist for vitamin B_6. This offers some hope for a therapeutic use, and some promising attempts have been made in experimental tumors,[25,29–36] with occasional clinical application. There are several practical difficulties, and no one is yet proposing an actual widespread clinical usefulness for this antagonist.

Mode of Action

Its mode of action, so far as analyzed, seems to be the following. 4-Desoxypyridoxine does not interfere with various aspects of vitamin B_6 formation or metabolism, illustrated in Figure 2, but it is itself phosphorylated, and while the phosphate does not displace pyridoxal phosphate from the enzymes once the latter has been firmly attached, still desoxypyridoxine phosphate can compete with pyridoxal phosphate when they both have an essentially equal chance of reaching the enzyme.[37]

In Figure 2, the site of action of desoxypyridoxine is not at *A* or *B*, but rather at *C*,

and biologically desoxypyridoxine + ATP forms desoxypyridoxine phosphate which becomes adsorbed to the enzyme and prevents pyridoxal phosphate from adsorbing, and thus activating, the enzyme. But both the phosphorylation (*B*) and the adsorption to the

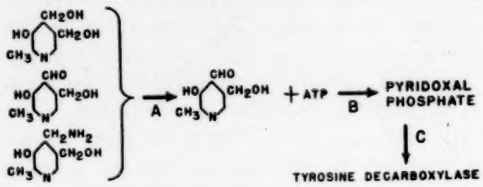

Fig. 2. Reactions involved in the activation of enzymes with members of the vitamin B_6 group.

enzyme (*C*) occur only when pyridoxal (for *B*) or pyridoxal phosphate (for *C*) are very low. This is probably why desoxypyridoxine is an active inhibitor essentially only at low levels of vitamin B_6. This type of study has been extended in two directions. First, the enzyme *B* in Figure 2 has been studied by Hurwitz.[11] This enzyme reacts with and phosphorylates a variety of substituted pyridines. It was shown, as illustrated in Figure 3, that 2-ethyl-3-amino-4-ethoxymethyl-5-aminomethyl pyridine inhibits pyridoxal phosphate formation from pyridoxal and ATP. It is certainly curious that a variety of more subtle changes do not produce inhibitory analogues but that changes are required in the 2, 3, 4 and 5 positions. In the same system, in the absence of pyridoxal, four compounds compete with pyridoxal phosphate after phosphorylation. They all contain the 5-hydroxy methyl group, the presumed site of phosphorylation. It is perhaps surprising that pyridoxine is such an inhibitor.

Second, Meister, Sober and Peterson[9] have employed crystalline desoxypyridoxine phosphate and pyridoxine phosphate[38] and have shown them to be capable of inhibiting transaminase when allowed to absorb to the enzyme before access to pyridoxal phosphate. The binding of inhibitors to the enzyme is considerably weaker than the comparable binding of pyridoxal phosphate based on dialysis experiments.

While the earlier studies of the activity of desoxypyridoxine are amply confirmed by these two independent studies, the inhibitory action of pyridoxine phosphate is not only surprising but rather makes one wonder about the significance of the studies on desoxypyridoxine. The various forms of vitamin B_6 differ in their ability to counteract desoxypyridoxine inhibition, which has led to a hypothesis that it interferes with the absorption of vitamin B_6.[39] Actually, the data would be equally compatible with any of a variety of other hypotheses. I think a way out of the dilemma posed by the inhibition observed by pyridoxine phosphate in rather purified systems would be the real possibility that an enzyme exists which will convert pyridoxine phosphate to pyridoxal phosphate and that this enzyme is present in the living cell. Thus pyridoxine phosphate which can be formed by an enzyme-catalyzed reaction between pyridoxine and ATP is converted to the active coenzyme (impossible with desoxypyridoxine): so the latter remains an inhibitor.

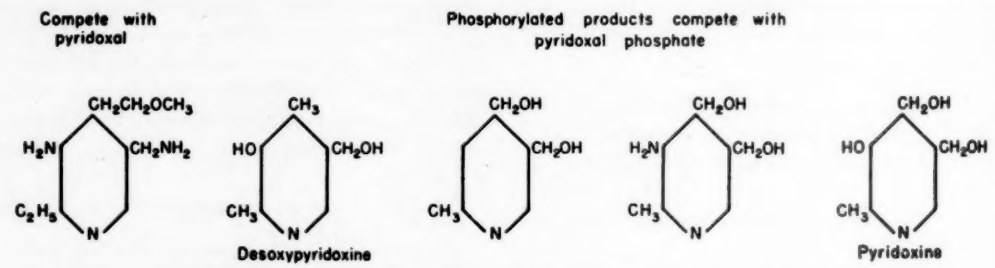

Fig. 3. Antagonists which act either by competing with pyridoxal or whose phosphates compete with pyridoxal phosphate.

Methoxypyridoxine

The methoxypyridoxine,[40,41,42] which has been studied less and not from the point of view of mechanism, and the 5-desoxy compounds are even less well explored.[43] As a matter of fact, for several years there has been relatively little widespread interest in vitamin B_6 or its antagonists, and relatively little fundamental work has been done. This attitude arose in part from the lack of spectacular therapeutic success except under restricted conditions and the general attitude that vitamin B_6 deficiency was not ever encountered in man, except under the most extreme circumstances. While the latter concept is at present undergoing a marked revision, to the extent, indeed, that perhaps what we call "normal" is borderline vitamin B_6 deficiency, it still remains that, from the point of view of a therapeutic agent, the fact that vitamin B_6 is concerned with so many basic reactions of amino acids renders it unlikely, at least according to theory, that sufficient specificity will be found to permit the specific therapy necessary. I have pointed out how what is known of the action of 4-desoxypyridoxine cannot be entirely explained by its action as a simple vitamin B_6 antagonist.

This would suggest that there are possibilities for therapy among compounds of this group, and that perhaps unexpected specificity may be found, especially if one does not lean too heavily upon vitamin B_6 antagonism *per se*. However, such studies remain for the future. Perhaps it is indicative that a clinically successful chemotherapeutic agent, isonicotinic hydrazide, is a vitamin B_6 antagonist.[44,45] Our own studies agree that with respect to tryptophanese,[44] isonicotinic hydrazide is an excellent antagonist of pyridoxal phosphate, although we do not, as is reported,[45] find that it antagonizes pyridoxal phosphate in amino acid decarboxylation. Its action in our hands[27] is not merely that of the hydrazide combining with the 4-aldehyde of pyridoxal phosphate. However, certainly isonicotinic hydrazide is known for its action in the tubercle organism,[46] and in this case its activity either in culture[47] or against the disease in the mouse is not reduced by vitamin B_6[47,48,49] and its action in the tubercle organism, therefore, appears to be based on some other property. Some of the side effects produced by isoniazid, however, are cleared up by the administration by pyridoxine,[48,49,50] when these are also cleared up by the withdrawal of isoniazid.

There is another even more surprising vitamin B_6 antagonist, and this is the pyrimidine moiety of thiamine. Injection of this substance causes convulsion in mice or rats, and this effect is completely eliminated by administration of vitamin B_6.[51] The phosphorylated pyrimidine seems to be a potent antagonist of pyridoxal phosphate.[52] A few other vitamin B_6 antagonists[4] have been reported to my knowledge, but little further has been done with them.

Conclusions

There seems to be a time and tide in scientific development, as there is alleged to be in the affairs of men, and with respect to vitamin B_6 we seem to be, after a period of relative quiescence, at the threshold of a resurgence of interest. At such a point it is frequently futile to discuss either past successes or failures or future possibilities, because a new concept or a new orientation may illumine with significance what is now darkened with neglect. One can sense, I think, that vitamin B_6 is at this stage, and I would suppose that in five years from now something of real clinical significance may emerge and a discussion of vitamin B_6 antagonism be thus of much more significance. At any rate, this is my picture of the mode of action and therapeutic implication of the vitamin B_6 antagonists known as of today, and I find it prudent to make no predictions for the future whatsoever, certain in the knowledge that no matter what one might predict at this point, experiment would be certain to demonstrate that the facts and their significance are otherwise.

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Pantothenic Acid Antagonists

By O. D. Bird, PH.D., E. L. Wittle, PH.D., R. Q. Thompson, PH.D., and Virginia Mims McGlohon, B.S.

One of the earliest planned antimetabolites was pantoyltaurine, reported by Snell in 1941[1] and obviously patterned after the sulfonamides, whose action had only recently been shown to be reversed by *p*-aminobenzoic acid. Pantoyltaurine was a competitive inhibitor of pantothenate in micro-organisms requiring pantothenic acid as a growth factor, and was claimed at first to produce a deficiency of this vitamin in animals, although this could not be confirmed. Subsequently a great many antimetabolites based on pantothenic acid have been made and found generally to follow this same pattern: they inhibit micro-organisms requiring pantothenate as a growth factor but are relatively inactive against others and, with one exception, none has produced a pantothenate deficiency in animals. The recent elucidation and synthesis of pantethine[2] and the availability of micro-organisms using pantethine and pantothenic acid with varying degrees of efficiency presented an opportunity to study new antimetabolites of this vitamin.

Pantoyltaurine and its amides, which correspond even more closely in pattern to the sulfonamides, have been studied extensively in several species, including animals.[3,4] This was before it was fully realized what drawbacks accompany the use in animals of antimetabolites based on vitamins. Some of these compounds successfully reduced experimental streptococcus infections in certain animals, but the required dose was impractical. The results were better in rats than in mice because the blood concentration of pantothenic acid was much higher in mice.

Since that time, many antagonists of pantothenic acid have been prepared, some of them having interesting characteristics. The majority of them have resulted from alteration in the β-alanine part of the molecule; some have involved changes in the pantoyl portion, and in a few, both parts of the molecule were altered. An interesting but not very effective antagonist, pantothenyl alcohol, was introduced by Snell in 1945.[5] It was active *in vitro* against micro-organisms requiring pantothenic acid, but on being ingested by animals was converted to pantothenic acid and had the usual vitamin activity of this compound. Pantothenone, a phenylketonic derivative of pantothenic acid, was studied by Woolley,[6] who found it to inhibit many species, but its effects were reversed by pantothenic acid only in those organisms requiring an exogenous source of this vitamin. This antagonist, as well as some of the substituted amides of pantoyltaurine,[7] have shown activity against malarial infections.

A Pantothenic Acid Antagonist

A potent antagonist of pantothenic acid, made by substituting methyl for hydrogen in the terminal group of the pantoyl part of the molecule, was studied by Drell and Dunn in 1946.[8] The compound was called ω-methylpantothenic acid and had been prepared and briefly studied by a graduate student in Henze's laboratory at the University of Texas three years before. He had noted that it lacked vitamin activity but did not detect its inhibitory action. In their first study of this compound, Drell and Dunn reported that it was a competitive inhibitor of pantothenate in many species of lactobacilli requiring this vitamin; later they reported[9] success in producing a pantothenate deficiency in mice and rats which was also competitive in nature. Shils[10] has since observed a decreased ability

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of rats to acetylate sulfanilamide when receiving ω-methylpantothenate. Recently, Bean and Hodges[11] have reported success in producing a pantothenate deficiency in four volunteer human subjects by giving them ω-methylpantothenic acid. This seems to be the first report on the intentional development of a pantothenate deficiency in humans. Since this work with humans, as well as Drell and Dunn's experiments with rats, have given results contrary to our limited experiments with rats, to be described later, it may be pertinent to comment on the Bean and Hodge report. These workers fed a purified and chiefly synthetic diet, supplemented with vitamins other than pantothenic acid, to their three volunteers, following preliminary experiments with one subject. The diet alone admittedly caused some biochemical alterations. This diet, supplemented with pantothenic acid, was given during a 12-day preliminary period. During the next 35 days the pantothenic acid was replaced with 0.5 gram ω-methylpantothenic acid daily. Then a period was started during which the antagonist was continued but it was supplemented with 4-gram daily doses of pantothenic acid. After six days on this regime the subjects were in such bad shape that the schedule was abandoned and emergency therapy of unstated nature instituted. It was assumed that the symptoms exhibited by the subjects were caused by the antagonist, although no individuals were maintained on the control diet during the experimental period; furthermore, six days of treatment with the relatively huge dose of 4 grams pantothenic acid, compared to 0.5 gram antagonist, did not counteract the effects of the latter. This would tend to cast doubt on the specificity of the symptoms which were reportedly due to the antagonist.

Experimental

The generally accepted microbiological procedures were used in our experiments with antagonists of pantothenic acid. Cultures were grown in Evelyn test tubes containing 10 ml medium, so that the amount of growth could be determined directly by reading in the colorimeter. Inocula were carefully standardized by turbidity readings. The inhibition index was determined by dividing the amount of metabolite needed for complete growth into the amount of antagonist required to produce half inhibition.

The compound studied most extensively was ω-methylpantethine. The inhibition indices obtained for several preparations of this compound are compared in Table I. It may be noted that the indices obtained after 18 hours' incubation vary, and even some of the preparations which had correct analytical figures gave low microbiological activity. As was the case with pantethine, which is also noncrystalline, the only meaningful criterion of purity available was the microbiological assay. Another characteristic which all these preparations of ω-methylpantethine had, in varying degrees, was a tendency to lose their inhibitory activity after increased incubation time.

TABLE I

Inhibition Indices for Different Lots of ω-Methylpantethine on *L. Helveticus*

Preparation Number	Origin	Inhibition index			
		18 hr	24 hr	40 hr	72 hr
03983	Snell	1400	1700	>4000	—
79-2147	Wittle[1]	700	800	>4000	—
80-2147	Wittle[2]	330	700	>4000	—
3771	Cavalla	1200	1200	>4000	—
16-2357	Wittle[3]	100	100	250	850

[1] Made with lactone from California Foundation for Biochemical Research. Analyzed correctly for C, H, N, S.

[2] Made with unreacted lactone recovered from 79-2147.

[3] Made with lactone prepared at Parke-Davis Laboratory.

In Table II are given inhibition data for our best preparation of ω-methylpantethine (16-2357) compared to data obtained with ω-methylpantothenate, acting against a variety of bacteria with different relative requirements for pantothenate and pantethine. The relative amounts of the two forms of the vitamin needed for growth of each organism are shown in the upper two lines of data. Below this are the inhibition indices produced when the antagonist was ω-methylpantothenate and the metabolite was alternately pantothenate and

pantethine for each organism. At the bottom of the table, similar data are presented for experiments in which the antagonist was ω-methylpantethine. It is difficult to find any pattern in these inhibition data. For *L. helveticus* and *S. faecalis*, ω-methylpantothenate is much more active when the metabolite is pantothenate, although for *L. arabinosus* and *L. citrovorum*, the form of the vitamin in the medium on which they grow makes little difference in the amount of inhibitor required for half maximum inhibition. When the antagonist is ω-methylpantethine, inhibition is better for all four organisms when growing in medium containing pantethine. So, in general, the results indicate that ω-methylpantothenate interferes with the utilization of pantothenate, while ω-methylpantethine interferes with the utilization of pantethine.

ω-Methylpantethine was found to be a competitive antagonist of *L. helveticus* against pantethine over the range investigated. The indices found when 0.25, 0.5, and 1 μg/10 ml amounts of pantethine were present were 320, 320, and 380, respectively. However, when *L. citrovorum* was inhibited by ω-methylpantethine (Table III), the inhibition when the metabolite was pantothenic acid was competitive but not very strong. The inhibition against pantethine was much stronger but noncompetitive. Pantethine produces the effective type of reversal in which an increase of metabolite concentration takes more than an equivalent increase in antagonist to overcome it.

Pantothenic acid is normally eight to ten times more active for *L. arabinosus* than pantethine. However pantetheine, the reduced sulfhydryl form, is several times more active (Table IV) than pantethine. This is similar to the relative growth-promoting activities of these two compounds previously reported[12] for *A. suboxydans*. The same phenomenon seems to play a part in the inhibition of *L. arabinosus*

TABLE II

Inhibition of Several Organisms with Different Relative Requirements for Pantothenate and Pantethine by ω-Methylpantothenate and ω-Methylpantethine

	L. helveticus		*S. faecalis*		*L. arabinosus*		*L. citrovorum*	
	PA*	PAN†	PA	PAN	PA	PAN	PA	PAN
Relative requirement for growth	100	1	1	1	1	10	1	100
	Inhibition index							
ω-methylpantothenate	500	52,000	500	>20,000	300	30	250	50
ω-methylpantethine	2500	100	>20,000	6000	20,000	40	9000	80

* PA = Pantothenic acid.
† PAN = Pantethine.

TABLE III

Inhibition of *L. citrovorum* by ω-Methylpantethine and Reversal by Pantothenic Acid vs. Pantethine

Ratio Inhibitor/Vitamin*	Inhibition index 18 hr	24 hr	40 hr	72 hr	Type of inhibition
μg/10 ml					
PA					
9000/1	9000	11,000	15,000	20,000	Competitive
18,000/2	9000	10,000	20,000	20,000	
PAN					
750/100	7.5	10	10	20	Noncompetitive
3500/200	17.5	40	50	80	

* Giving half inhibition.

TABLE IV

Growth Response of *L. arabinosus* to Pantetheine vs. Pantethine

	Required for ½ maximum growth	
	24 hr	40 hr
	μg/10 ml	μg/10 ml
Pantothenic acid	0.08	0.08
Pantetheine (reduced)	0.20	0.10
Pantethine (oxidized)	0.70	0.33

TABLE V.

Inhibition Index of ω-Methylpantothenic Acid and Reduced vs. Oxidized Pantethine on *L. arabinosus*

Vitamin	Ratio Inhibitor/Vitamin	Inhibition index 18 hr	40 hr	72 hr
	μg/10 ml			
PAN	40/2	20	50	50
Reduced PAN	300/2	150	150	250
PA	150/0.5	300	1200	2000

by ω-methylpantothenate (Table V). The inhibition ratio for pantetheine, the reduced form, is about eight times higher than for pantethine, possibly indicating that the reduced form of the vitamin enters the cell to a greater extent and therefore requires more of the antagonist to neutralize it.

the first two, which have only a single alteration, and that all of them are more effective antagonists against the utilization of pantethine than against the utilization of pantothenate. Further investigation of these compounds might produce interesting results.

Elion and associates[13] have proposed a

TABLE VI

Inhibitory Effect of Analogues of Pantethine Containing Modifications of One and Two Parts of the Molecule on *L. arabinosus* and *L. helveticus*

Inhibitor	Organism	Metabolite	Inhibition index 18 hr	24 hr	40 hr	72 hr
Pantothenylaminoethane	*L. helv.*	PAN	80	—	150	400
Pantothenylaminoethanol	*L. arab.*	PA	40	—	60	100
	L. arab.	PAN	8	—	8	8
	L. helv.	PAN	220	—	220	220
ω-Methylpantothenylaminoethane	*L. arab.*	PA	1400	>2000	>2000	>2000
	L. arab.	PAN	400	400	400	400
	L. helv.	PAN	70	75	270	450
ω-Methylpantothenylaminoethanol	*L. arab.*	PA	1400	>2000	>2000	>2000
	L. arab.	PAN	400	400	400	400
	L. helv.	PAN	500	500	700	800

The inhibitory activity of pantethine analogues in which the β-mercaptoethylamine part of the molecule is replaced by aminoethane and aminoethanol is shown in Table VI. Also indicated is the activity of these compounds which have been further modified by the substitution of methyl for hydrogen at the terminal carbon atom of the pantoyl part of the molecule. In the case of three of these compounds, the only ones investigated in this respect, their activity against *L. arabinosus* growing on pantethine remained constant over a long incubation period. Both ω-methyl-substituted compounds were less active than pantothenylaminoethanol. The general impression is that the hybrid analogues with double substitutions have no advantage over

method for determining synergism between purine antagonists. Table VII shows data obtained on *L. citrovorum* when this method was applied to the combined action of ω-methylpantothenate and ω-methylpantethine in various concentrations. Each tube contained 1 μg pantothenate as growth factor. The results are expressed as milligrams of dried cells in each culture tube, which makes it easier to visualize the synergistic effect of these two antagonists. When the resulting data are calculated by the formula derived by Elion *et al.*, as indicated above, a value of 0.61 is obtained. This suggests a considerable degree of synergism between these two compounds, since a figure less than 1 indicates synergism and more than 1 antagonism. By this same procedure,

TABLE VII

Inhibitory Effect of Combinations of ω-Methylpantethine and ω-Methylpantothenic Acid on *L. citrovorum* with Pantothenic Acid as Growth Factor

mg ω-methylpantethine per 10 ml	μg ω-Methylpantothenate per 10 ml				
	0	75	150	225	300
	mg dry cells per 10 ml				
0	3.8	2.2	1.4	0.9	0.5
2	2.8	0.8	0.5	0.2	0.3
4	1.8	0.6	0.2	0.05	0.0
6	1.2	0.6	0.1	0.0	0.0
8	0.9	0.3	0.1	0.0	0.0
10	0.6	0.2	0.1	0.0	0.1

a combination of ω-methylpantothenate and ω-methylpantethine produced an index of 0.58, indicating synergism, when acting on *L. citrovorum* with pantethine as the growth factor, while ω-methylpantethine and pantothenylaminoethanol acting on *L. helveticus* with pantethine as growth factor were antagonistic (coefficient 1.7).

or pantethine. Amounts of inhibitor up to 200 times the pantothenate or pantethine given had no antagonistic effect. Pantothenylaminoethane and pantothenylaminoethanol were each given intraperitoneally by injection at a level of 6 mg per day to deficient rats. Both these compounds produced a growth response equal to that obtained with an equivalent amount of pantothenate, indicating that these antagonists were evidently cleaved to pantothenate by the rat under the test conditions.

As indicated in a recent publication from our laboratory,[15] pantothenylcystine failed to give significant pantothenate activity in either rats or chicks, given orally or parenterally. Nor was there any detectable inhibitory activity shown by this compound, although, of course, it was given in small amounts.

Experiments were conducted to try to find why the inhibited cultures of the micro-organisms studied grew out after long incubation. Since ω-methylpantethine contains peptide bonds, a possible reason why cultures inhibited by this compound grew out might be the gradual hydrolysis of this inhibitor down to ω-methylpantoic acid which does not inhibit *L. helveticus*. *L. helveticus* was inoculated into medium containing 1000 μg ω-methylpantethine per 10 ml. After 18 hours' incubation this preparation gave an inhibition index of 200. Continued incubation for a total of 72 hours resulted in good growth of the culture. The cells of the 72-hour culture were then centrifuged and the inhibition index of the supernatant determined by inoculating dilutions of it with fresh inoculum. The index was 240, showing that the ω-methylpantethine was still intact after the *L. helveticus* cells had grown out in its presence.

TABLE VIII

Pantothenate Antagonist Studies in Rats

Compound	Level and how given	Type of diet	Result
ω-Methylpantothenate*	200x PA or PAN, orally	Low level PA or PAN	No antagonism or activity
ω-Methylpantethine	0.05% orally	PA-deficient	No antagonism
Pantothenylaminoethane	6 mg/day IP	PA-deficient	PA activity
Pantothenylaminoethanol	6 mg/day IP	PA-deficient	PA activity
Pantothenylcyst(e)ine	68 μg/day IP	PA-deficient	Insignificant activity, no antagonism

* Obtained from California Foundation for Biochemical Research.

Since Drell and Dunn[9,14] have reported the production of a pantothenate deficiency in mice and rats fed ω-methylpantothenate, we attempted a similar experiment in which rats were given a pantothenate-deficient diet to which, after 3 weeks, 0.05 per cent ω-methylpantethine was added. The data for this and similar experiments are summarized in Table VIII. This amount of antagonist did not hasten the further development of the deficiency. The test was run in this way because it seemed the most sensitive method by which an *in vivo* effect might be shown. The calcium salt of ω-methylpantothenate, the same compound reported on by Drell and Dunn, was also studied. In this case rats were made pantothenate-deficient, then fed various ratios of inhibitor and either calcium pantothenate

Another possibility which suggested itself was that resistance develops suddenly, a situation which has been observed with tubercle bacilli inhibited by isoniazid. To test this, a culture of *L. helveticus* was incubated 40 hours in the presence of ω-methylpantethine. It was then growing rapidly. A washed inoculum from this culture was added to medium containing the same antagonist, with the thought that it might have become adapted to the antagonist and would grow out more rapidly than an inoculum of a like number of uninhibited cells in the same medium. But the antagonist-treated inoculum actually grew out more slowly. In fact, even when the inoculum from medium containing antagonist was transferred to normal medium with no antagonist, it took about 40 hours to grow out, whereas normal inoculum grew out in 10 to 12 hours.

The following experiment was then carried out. A large inoculum of *L. helveticus* cells which had grown out after 40 hours' incubation in the presence of ω-methylpantethine was transferred to normal medium. After 32 hours' incubation, when the cells were growing quite rapidly, they were transferred to medium containing the antagonist and incubated for 16 hours. A small inoculum of these cells grew out at the same rate as cells from normal medium when transferred to medium containing no antagonist, while cells which had been exposed only once to the antagonist grew out as slowly as before. We interpret this behavior as an indication that cells which grow out in the presence of this pantothenate antagonist gradually become impermeable to it and, when transferred to normal medium, are unable to grow immediately because of the concentration of antagonist still inside the cells. After another passage through normal medium, however, a subsequent exposure to the antagonist does not harm the cells and leaves them in a condition to grow at the normal rate when placed in medium containing no antagonist.

Discussion

With some exceptions, notably in the cases of pantothenylaminoethanol, ω-methylpantothenylaminoethane, and ω-methylpantothenylaminoethanol acting upon *L. arabinosus* with pantethine as a growth factor, all the microbiological inhibitions studied resulted in the organisms growing out after long incubations. This is contrary to the results reported by Drell and Dunn,[9] who obtained low inhibition indices for ω-methylpantothenate in several micro-organisms even when calculated after 72 hours' incubation.

The experimental results obtained with animals in our laboratory are also in conflict with those of Drell and Dunn, and, at least by implication, with those of Bean and Hodges[11] in humans. Drell and Dunn produced a pantothenate deficiency in mice and rats with ω-methylpantothenate which could be reversed competitively with pantothenate. In our experiments, neither ω-methylpantothenate nor ω-methylpantethine produced any antagonism which could be observed in terms of growth. Bean and Hodges produced a condition reputedly due to pantothenate deficiency. However, the small amount of antagonist required (only 0.5 g per day for an adult) and the fact that the effect was not reversed in six days by enormous doses of pantothenate, leave some doubt as to the true nature of the condition these workers observed.

The inability of rats to utilize pantothenylcystine indicates that they are unable either to utilize this compound to synthesize coenzyme A or to degrade it to pantothenic acid. This is contrary to what has been reported for both bacteria and liver supernatant.

Summary

Different preparations of ω-methylpantethine varied greatly in their inhibition indices. These differences could be detected by microbiological tests, but not by chemical analyses or physical tests.

The pantothenic acid antagonists reported here were only temporary inhibitors of the growth of most micro-organisms investigated.

ω-Methylpantothenate tends to inhibit the utilization of pantothenate, while ω-methylpantethine tends to inhibit the utilization of pantethine.

None of the antagonists investigated produced a pantothenate deficiency in rats. ω-Methylpantothenate and ω-methylpantethine

were inactive both as antagonists and as growth factors replacing pantothenic acid. Pantothenylaminoethane and pantothenylaminoethanol were good antagonists of pantothenate in micro-organisms, but had pantothenate activity in rats.

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A Naturally Occurring Antimetabolite of Methionine *in the Causation of a Disease*

By D. W. Woolley, PH.D.

It is becoming increasingly clear that antimetabolites are not merely the creation of organic chemical laboratories. On the contrary, it emerges that Nature knows about the phenomenon of antimetabolites, and many of her creatures make them and use them in normal physiological processes. This concept arose more than ten years ago, but gained acceptance so slowly that even now it is not believed in many quarters. This has been partly because the numbers of naturally occurring and recognizable antimetabolites are not nearly so large as the synthetic ones. Then, too, earlier notions about the modes of action of some of the natural members have tended to persist and to distract attention from the fact that these substances really are antimetabolites.

One of the first cases of a naturally occurring antimetabolite was discussed in a paper at the first meeting of this society about ten years ago here in New York. This was the case of the pellagragenic agent of corn which is an antagonist of nicotinic acid. However, this case leaves much to be desired because the exact structure of the antagonist is not yet established.

Today, I would like to tell you about a more recently discovered and clearer example of a naturally occurring antimetabolite. This is the toxin of *Pseudomonas tabaci,* which is an antimetabolite of methionine.

Basic Principles

First, let us briefly review what is meant by an antimetabolite.[1] Such a compound is a structural analogue of some vitally essential component of living things. However, the structural analogy does not, of itself, make a compound an antimetabolite. In addition, the analogue must be able to antagonize the action of the metabolite. In other words, it must be able to exclude its related essential metabolite from the specific cellular reactions for which the latter is designed. A deficiency of this essential metabolite is thereby created. The antimetabolite competes with the metabolite when the latter is serving as a substrate in an enzymic or semi-enzymic reaction. Because it preferentially attaches to the enzyme, it is able to prevent the formation of the normal product of the reaction. In this way the deficiency is produced. The structural analogy of the antimetabolite to the metabolite is presumably what allows it to make this attachment. Being so nearly like the metabolite, the antimetabolite fits into the specific receptor of the enzyme, much as the metabolite itself does.

Now let us consider the toxin of *Pseudomonas tabaci.* There is a disease of tobacco, and of certain other crops, which is caused by a pathogenic bacterium. This organism, *Pseudomonas tabaci,* enters the leaves of the plants, grows there, and produces lesions which consist of necrotic spots surrounded by yellow halos. The disease can spread so rapidly through a field that it is called wildfire disease. The lesions are caused by an extracellular toxin which is formed by the pathogen. Thus, if the organism is grown in the laboratory, and a filtrate of the culture is made, this filtrate will, when it is applied as a drop to a tobacco leaf, reproduce the signs of the disease (see Figure 1). The amount of toxin in a drop can be measured by determination of the diameter of the halo. Within certain limits, the larger the spot, the more toxin was present. This has been the basis for quantitative assay of the toxin. This method has

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been used by Dr. A. C. Braun who has done much of the biological investigation of this disease, and with whom we have had the good fortune to collaborate.[2]

Dr. Braun found that a unicellular plant, viz., *Chlorella vulgaris*, was also susceptible to this toxin. In this alga, he found that the growth-inhibiting property of the *Pseudomonas* toxin could be reversed by methionine. This action was quite specific in that no other amino acid was effective. Furthermore, the antagonism between methionine and the toxin was of the reversible and competitive kind. This character of the antagonism is always compelling evidence, although we must not suppose, as some do,[3] that it is the *sine qua non* of antimetabolite action. In fact, although the toxin was a competitive antagonist of methionine in *Chlorella*, in tobacco plants this amino acid was not able to protect against the toxin. Much experimentation has gone into the understanding of this failure, and it now appears likely that extraneous causes of penetrability and toxicity of methionine in the tobacco tissue becloud the picture.

Because of the clear-cut results with *Chlorella*, the working hypothesis was formed that the toxin was an antimetabolite of methionine and that it owed its pathogenic action to the induction of a specific deficiency of this amino acid. To test this hypothesis it was necessary to isolate the toxin in pure form, and to establish its chemical structure. This has been done, and the idea that it would be analogous to methionine has been verified.

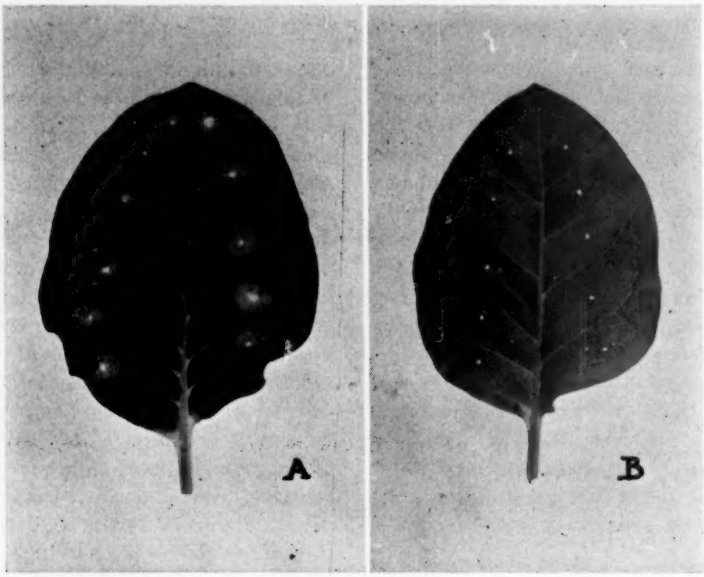

Fig. 1. Tobacco leaves inoculated with (*A*) sterile wildfire toxin; (*B*) control media.

Before beginning these chemical studies, we had a further indication of the soundness of the working hypothesis. This was that a synthetic antimetabolite of methionine would reproduce the disease in tobacco plants. This synthetic compound was methionine sulfoximine, which, at that time, had just been shown to be the causative agent of "running fits" in dogs. You will recall that this antimetabolite arises in wheat flour when the latter is treated with nitrogen trichloride. It is clearly a structural analogue of methionine, and many of its toxic effects can be overcome by this amino acid. When methionine sulfoximine was applied to tobacco leaves, it produced the typical lesions of wildfire disease. It was of much interest, however, to find that methionine would not prevent these lesions any more than it would prevent those called forth by the

Pseudomonas toxin. If *Chlorella* instead of tobacco was the test plant, then, just as with the toxin, the action of methionine sulfoximine could be overcome. The structures of methionine and of methionine sulfoximine are shown in Figure 2.

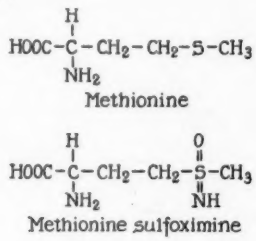

Figure 2

Isolation

The isolation of the *Pseudomonas* toxin proved to be very difficult. This was because of its instability, especially to very weak alkali, and also because of the fact that it was a water-soluble substance quite insoluble in almost all organic solvents. Furthermore, it was present in very small amounts in the culture filtrates. A method of isolation was finally devised[4] which is summarized in Figure 3. It consisted of adsorption of the toxin to alumina under acidic conditions in methanolic solution, and elution with acidified water. The material so obtained was then separated on large columns of powdered cellulose. It had been found that the toxic activity was associated with a substance which gave a ninhydrin test, and which had an R_F on paper chromatograms of 0.26 in a mixture of propanol and water. By use of these properties, tests were devised for locating the position of the toxin in effluents of the columns. Results so obtained were always verified by assays on tobacco leaves.

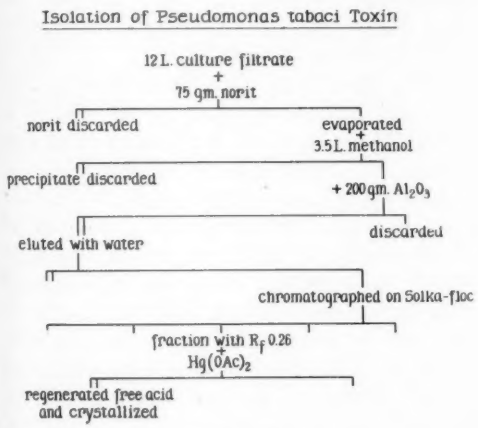

Figure 3

The active fraction from the chromatographic columns was contaminated with an unknown organic acid. In order to bring about final purification of the toxin, it was precipitated as the mercury salt, and the free toxin was then regenerated and caused to crystallize. The yield was about 4 mg per liter of culture.

The isolated toxin was very active in causing the wildfire lesion of tobacco leaves. Thus, it was detectable at about 0.005 μg.

Chemical Structure

The chemical structure of the pure toxin was established by suitable analyses and degradations to known compounds.[5,6] The main features of the degradations are shown in Figures 4 and 5. The proposed structure of the toxin is also shown in Figure 5. The elementary analyses revealed that the empirical formula was most probably $C_{10}H_{16}O_6N_2$, and that the toxin was α-amino acid. One of the two nitrogen atoms occurred as a free α-amino group, and the other one was an acylated amino group.

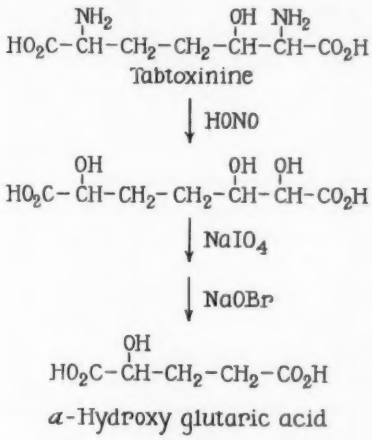

Figure 4

Complete hydrolysis with acid gave rise to lactic acid plus a new amino acid which had previously not been known. This new compound contained both of the nitrogen atoms of the original toxin and seven of the ten carbon atoms. It was identified as α,ϵ-diamino-β-hydroxypimelic acid by suitable degradations to glutamic acid with periodate, and to α-hydroxyglutaric acid with nitrous acid, hypobromite and periodate (see Figure 4). This new amino acid was named tabtoxinine.

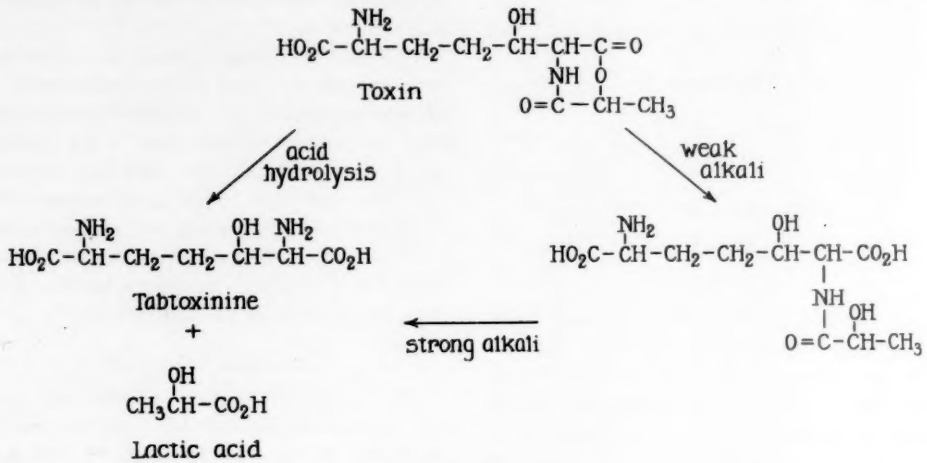

Figure 5

With these two products of complete hydrolysis, viz., lactic acid and tabtoxinine, all of the carbon and nitrogen atoms were accounted for, and the problem resolved itself into the question of how they were united in the toxin. Very mild alkali destroyed the biological activity of the toxin, and gave rise to a new compound which was isolated and identified as most probably α-lactyltabtoxinine. In other words, the lactic acid was the acylating group which bound one of the nitrogen atoms. The toxin itself was the lactone formed by ring closure of the α-carboxyl and the hydroxyl of the lactyl residue.

Although the formula shown in the figure seemed to be the most probable one for the toxin, there was still some little doubt about it. Thus, small amounts of formic acid were also recognized as a product of acid hydrolysis. At one time[7] it was felt that the acyl group was formyl rather than lactyl. However, a considerable amount of work all pointed to the conclusion that the formic acid probably arose from more profound decomposition of the recognized constituents. The proposed structure for the toxins seemed adequate to account for its chemical properties, but we must carry with us this slight reservation about its absolute correctness.

I fear that I may have bored many of you with this brief description of the chemical work. These days, many are content to buy a chemical compound from the supply houses, or to write to someone and request a gift. If these two easy procedures fail, the general reaction seems to be not to investigate the substance. Nevertheless, I feel that a knowledge of organic chemistry is at the very heart of the study of antimetabolites. From the very beginning, it has been necessary to synthesize all sorts of strange new compounds, for without them the whole field of antimetabolites would have been almost barren. If we rely on only the compounds we can buy or beg, I fear that our understanding of this whole subject will be considerably stunted. It is partly for this reason that I have ventured into a discussion of the organic chemistry of the *Pseudomonas* toxin. You will recognize, too, that the determination of its chemical formula was crucial to the concept outlined.

Relationships

Let us now ask how close the resemblance in structure is between methionine and the *Pseudomonas* toxin. The formulations of Figure 6 will illustrate this point. Consider first the relationship of methionine to the recog-

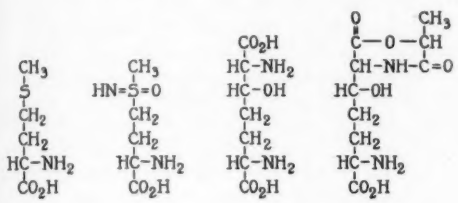

Figure 6

nized antimetabolite methionine sulfoximine. In passing from the metabolite to the antimetabolite the change has been to add an oxygen and an imino group to the sulfur. The analogy is clear. You will also recall that methionine sulfoximine is able to reproduce the signs of wildfire disease in tobacco leaves, although from a quantitative standpoint it is considerably less potent than is the natural toxin. The analogy of methionine sulfoximine to tabtoxinine is also rather close. The sulfur atom has been replaced by two carbon atoms (a well-known method of making antimetabolites[1]). The oxygen atom has gone with one of these carbons, and the nitrogen atom with the other. The double bonds have been reduced so that we have hydroxyl and amino rather than ketone and imino, and the methyl group has been correspondingly oxidized up to a carboxyl group. These changes bring us to tabtoxinine, the hydrolytic product of the toxin. The entire toxin results from the acylation of the amino group and closure of the lactone ring.

It is attractive to think that these last changes in structure are just a device for assurance of ease of permeability for the toxin. Thus, tabtoxinine has a highly ionized group at both ends of the molecule, and presumably, such highly polar substances find difficulty in penetrating cells. Methionine, on the other hand, is ionized at only one end. It would seem possible that the lactyl group on the amino group, and the lactone linkage of the carboxyl of the toxin are maneuvers to cancel out the charge at one end of the tabtoxinine molecule, and thus to allow the toxin to penetrate to its site of action in the plant. We plan to test this idea directly by synthesis of the appropriate derivative of tabtoxinine. Both tabtoxinine and the alkali-inactivated toxin have no toxic effects on tobacco leaves, and as you can see, these both have ionizable groups at both ends of the molecules.

Of course, one must not forget that the lactone grouping may play a role in anchoring the toxin to the methionine site in the tobacco cell. Such a chemically reactive grouping as this lactone would present possibilities for strongly binding the toxin by a covalent bond to this site in the victim. This may help to explain the very high potency of the toxin in contrast to methionine sulfoximine. It has been recognized previously[1] that the possession of chemically reactive groupings in addition to structural analogy to the metabolite, frequently makes the antimetabolite both highly potent and irreversible in its effects. and for quite evident reasons.

The fact that this toxin is an amino acid, while the bacterial toxins of microbial diseases of animals are usually proteins, is noteworthy. The plants do not have a circulatory system in any way comparable with the blood stream of animals. Consequently, large molecules travel slowly in plants. An invading parasite should find it advantageous to elaborate small, readily diffusible toxins rather than proteins. The case of the *Pseudomonas* toxin is not the first example to show that phytopathogens do this. The preceding case of the toxin of *Fusarium lycopersici*, which causes disease in tomato plants, was shown by work both in Switzerland and in our laboratory to be not a protein, but a modified tripeptide.[8,9] This tripeptide was shown to have a structural analogy, and an antagonistic action toward peptides which possessed strepogenin activity.

The *Pseudomonas* toxin now emerges as a second phytopathogenic substance which is a modified amino acid, and which is an antimetabolite of another essential metabolite,

viz., methionine. Some day it may be possible to show that the proteins which are the toxins of pathogenic bacteria in higher animals have a structural analogy to some specific and important proteins of the host, and that the toxins owe part of their poisonous effects to an antagonism to these constituents of the host.

Summary

To come back now from the realm of hypothesis, let us remember that a compound of chemical structure related to methionine occurs naturally as the toxin of the plant pathogen *Pseudomonas tabaci*. This toxin, which has been isolated in pure form and identified, reproduces the lesions of the disease. The same lesions can be caused by a synthetic antimetabolite of methionine. In the unicellular plant *Chlorella vulgaris*, the poisonous action of the *Pseudomonas* toxin can be overcome in a competitive fashion by methionine, and by no other known substance. For these reasons it seems rather clear that the *Pseudomonas* toxin probably owes much of its disease-producing properties to the fact that it is a naturally occurring antimetabolite of methionine.

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Folic Acid Antagonists

By JOSEPH H. BURCHENAL, M.D.

THE FACT that an analogue of folic acid, 4-amino pteroylglutamic acid (Aminopterin) would produce remissions in children with acute leukemia with some degree of regularity, as shown by Farber *et al.*[1] in 1948, gave a tremendous impetus to the chemotherapy of cancer and to the study of the various biochemical systems in which folic acid and certain closely related compounds are involved. Since Aminopterin or Methotrexate (4-amino N^{10} methyl pteroylglutamic acid) will in certain children temporarily eradicate all signs and symptoms of acute leukemia and temporarily return the patient to complete good health, we have been particularly interested in studying the mechanisms of the development of the resistance which finally sets at naught all therapeutic efforts with these compounds. In such studies a knowledge of the various systems which these compounds affect in bacteria, in mammalian tissues, and in the leukemic cell, as well as in the normal leukocyte, would be of great value. Information gained from such studies might eventually make it possible, first, to determine more precisely the mechanisms whereby these folic antagonists exert their effect on leukemia; second, to design compounds which might specifically damage the resistant leukemic cell; and third, to attempt rational combination therapy by the sequential blocking of the same metabolic pathway at several different levels in the hope of delaying or even preventing the development of resistance.

From the Chemotherapy Service, Memorial Center for Cancer and Allied Diseases, Division of Experimental and Clinical Chemotherapy, Sloan-Kettering Institute, and the Sloan-Kettering Division of Cornell University Medical College, New York, N. Y.

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Mechanisms of Action

It has been demonstrated by Nichol and Welch[2] and by Sauberlich[3] in the intact animal, by Nichol and Welch[2,4] in liver slices, and by Hendlin *et al.*,[5] Broquist *et al.*,[6] and Nichol *et al.*[7] in bacteria that the 4-amino derivatives of folic acid prevent the conversion of pteroylglutamic acid (PGA) to citrovorum factor (CF) and also in some way interfere with the action of CF itself. Hutchison *et al.*[8] and Nichol[9] using leukemic cells of mice *in vitro* have demonstrated that here, too, Methotrexate acts to prevent the conversion of PGA to CF. The more recent work of Blakley[10] has pinpointed the site of action of Aminopterin in a pigeon liver extract system as preventing the reduction of PGA to tetra hydro PGA. It has been shown by Greenberg[11] that citrovorum factor or a closely related compound is necessary for the transfer of one-carbon fragments such as formate or formaldehyde to both glycine amide ribotide, which is thus formylated to give the 8 carbon of the purine skeleton, and to 5-amino 4-imidazole carboxamide ribotide to form the 2 carbon of inosinic acid. His studies show that tetra hydro folic acid in the presence of ATP reacts with formate to form a compound which apparently after further conversion transfers its group to form the 2 or 8 position of the purine skeleton. Recent studies by Goldthwait[12] have shown that the effect of Aminopterin on this incorporation is directed equally against the 2 and the 8 carbon position. Similarly, a folic acid deficiency induced either by diet or by the 4-amino antagonists of folic acid has been shown to prevent the incorporation of formate into the 5-methyl position of thymine[13] and into the β carbon of serine.[14,15]

Such a deficiency also appears to prevent the incorporation of the α carbon of glycine into cysteine and glutamic acid[16] and into serine,[10] and the methyl carbons of betaine and methionine into choline and creatinine.[17] It also decreases markedly the choline oxidase activity of chick bone marrow[18] and of mitochondria of rat liver.[19] A lowering of DPN levels in rat liver after Aminopterin,[20] a lowered coenzyme A content of folic-deficient chick and rat livers,[21] and a lowered ability of folic-deficient rats to oxidize injected formate[22] have been reported.

Classification

There are five main groups of folic acid antagonists which I would like to consider this morning. The first class, exemplified by 9-methyl or 10-methyl pteroylglutamic acid, inhibits the growth of certain bacteria and animals[23–26] and can be reversed competitively by PGA. They prevent the conversion of PGA to CF in bacteria but do not compete with CF.[27] The second class of antagonists, of which 4-amino N^{10} methyl pteroylglutamic acid (Methotrexate) is a good example, inhibits the growth of bacteria,[5,7,28,33,83] and in *Streptococcus faecalis,* for example, this inhibition can be reversed by folic acid at low levels of the antagonist.[32] Much higher doses of PGA are necessary, however, as the concentration of the antagonist rises. These substances are reversed competitively by CF and noncompetitively by thymine and thymidine in *Leuconostoc citrovorum.*[34] In animals their toxic effects can be prevented only at very low levels by PGA,[32] but the toxic action of even massive doses of the antagonist can be prevented competitively by CF, both in animals[2,34,35] and in man.[36] As mentioned previously, these antagonists block utilization of CF competitively. Much of the ability of PGA to prevent toxicity of the folic acid antagonists in the mouse depends on administration of PGA sufficiently prior to the folic acid antagonist to allow for the conversion of PGA to CF before the injection of the folic acid antagonist.[37] Studies by our group have shown that, in a patient, as little as 3 mg daily i.m. of CF can prevent the toxic effect of as much as 45 to 60 mg daily by mouth of Methotrexate over a three-week period.[36] Thymidine also reverses the inhibitory effects of the 4-amino PGA's in the chick embryo.[38]

Another class of folic acid antagonists consists of the 2,4-diamino pteridines, which also act as antagonists of folic acid in bacteria,[39–41] but do not seem to be effective against mouse leukemias sensitive to Methotrexate[42] and have not been tried clinically. A fourth group, the diamino-dichlorophenyl pyrimidines, act in much the same way as the 4-amino derivatives of PGA.[43] They seem to compete even more with CF than do the 4-amino PGA's, in that in *S. faecalis* the inhibition produced by the pyrimidines is competitive with CF only over a very narrow range of concentration, whereas 4-amino N^{10} methyl pteroylglutamic acid is competitive with CF over at least a 200-fold concentration range.[44] The inhibitory effect in *L. citrovorum* can be reversed only moderately well by CF, and the toxicity in animals and man can be reversed by CF only at or just above the toxic level.[45,46] The dihydrotriazines synthesized by Modest,[47,48] and studied by Foley,[49,50,51] appear to act as noncompetitive inhibitors of folic acid and CF in *S. faecalis* and of CF in *L. citrovorum.* There is reported to be no significant cross resistance between these compounds and the diamino dichlorophenyl pyrimidines or the 4-amino antagonists of folic acid.[51]

It is of considerable interest that among the five classes of folic acid antagonists, only the 4-amino derivatives of PGA seem to have a selective effect on leukemia in children, in that they will produce remissions without concomitant toxicity. It may be that certain differences in the mode of action of these five groups of compounds will help to explain why Methotrexate affects the leukemic cell at doses which do not damage the normal cell.

In *Tetrahymena pyriformis (geleii)*, Kidder and Dewey showed that 4-amino N^{10} methyl PGA (Methotrexate) had a marked inhibitory effect on the growth of this organism,[52] but a chromatographically purified sample of the closely allied compound, 4-amino PGA (Aminopterin), had neither inhibitory nor growth-promoting effects.[53] It is of interest

that a 4-hydroxy compound, 3′,5′ dichloro N^{10} methyl PGA, was almost as potent as Methotrexate, the best of the 4-amino compounds against this organism.[54]

In intact animals, as in bacteria and protozoa, the 4-amino derivatives of folic acid exert a marked effect at relatively low doses. The development of *Drosophila melanogaster* larvae,[55] the growth of the four-day-old chick embryo,[56,57] and the formation of the blood islets of the yolk sac[58] are inhibited by the 4-amino derivatives of folic acid, and fetal abnormalities and fetal death have been produced in rats[59] and in patients.[60] In the presence of folic acid antagonist, estrogens do not stimulate the growth of the oviduct of the newly hatched chick[61] or the uterus of the weanling rat.[62] Similar observations have been made on the oviducts of newly metamorphosed frogs.[63] After a large single dose of Aminopterin, rats appear relatively normal for the first 24 hours after injection. Weakness and anorexia then appear, and at 48 hours a severe diarrhea develops, with death occurring within 72 to 96 hours. At autopsy, granulocytopenia, associated with degeneration and hypoplasia of the bone marrow and extensive degenerative changes in the small and large intestines and moderate damage to lymphatic tissues, are the chief findings.[64] In dogs, a somewhat similar situation occurs.[65] Although the action on lymphoid tissue of the 4-amino antagonists of folic acid are to some degree dependent on intact adrenals,[66,67] Aminopterin will produce bone marrow injury in the rat in the absence of the abdominal viscera, including the liver, kidneys, and adrenals.[68]

Weir, Welch and Heinle[69] first reported the effects of folic acid antagonists on the survival of mice with transplanted leukemia. They employed crude X-methyl folic acid, a purified folic-free diet, and sulfasuxidine to produce an increase in survival time of Ak mice with transplanted leukemia. Subsequently Kirschbaum,[70] Law,[71] and our group,[42,72] and many other investigators have shown that many, but by no means all, strains of transplanted leukemia in mice will respond to Aminopterin or Methotrexate. Myeloid strains in particular seem to be refractory to anti-folic therapy.[70,72] The anti-leukemic effects of these compounds can be demonstrated in adrenalectomized as well as intact animals.[71] Although these compounds have no effect on the survival time of transplanted leukemia when administered late in the disease, even a small dose of Methotrexate will rapidly reduce the total leukocyte count in mice with advanced Ak4 leukemia within 24 hours.[73] Despite the doubling or tripling of survival time of Ak4 leukemia caused by tolerated doses of the 4-amino antagonists of folic acid, these compounds, even when given at 50 to 100 times the acute LD_{50} dose, do not demonstrate the sterilizing effect on leukemic cells that is shown by HN2 and TEM at as little as 5 times the LD_{50} dose.[74] The 4-amino derivatives of folic acid also have slight or no apparent effect on normal mouse epidermis or Sarcoma 180 cells in tissue culture.[75] Using cells of a Methotrexate-sensitive strain of Line I mouse leukemia grown in tissue culture, Kieler and Kieler have demonstrated an inhibition of mitotic activity by Methotrexate at relatively high doses,[76] and Jacobson has shown a somewhat similar anti-mitotic effect in human and mouse bone marrow.[77]

The anti-leukemic as well as the toxic effect of the folic acid antagonists can be reversed by PGA if given long enough before the injection of the antagonist and in large enough doses, or it can be completely reversed by the simultaneous administration of CF.[78,79,80]

Clinical Experience

Since the original studies of Farber,[1] many patients with acute leukemia have been treated with the folic acid antagonists, and the overall results of therapy during the first two or three years[81] showed that 68 per cent of 425 children with acute leukemia received considerable clinical benefit from the administration of these agents.[82] Good hematological remissions occurred in 30 to 50 per cent of the children, with marrows returning to essentially normal morphology and function.

The folic acid antagonists are generally given clinically by the oral route, with the dose of Methotrexate ranging from 2.5 to 5.0 mg daily. Absorption is rapid, with blood levels

of 50 mγ per ml being detectable 15 minutes after the ingestion of 5 mg of Methotrexate on an empty stomach, with top levels of approximately 100 mγ/ml being attained 30 to 60 minutes after ingestion.[83] In patients under treatment with Aminopterin or Methotrexate in excessive doses, toxic effects such as ulceration of the buccal mucosa, abdominal cramps, diarrhea, and gastrointestinal ulceration and bleeding may occur, but with careful handling remissions in the early stages of the disease can usually be achieved without toxic manifestations.[84] Eventually, however, as the patient's disease becomes more resistant to the therapy, it will almost always be necessary to push the dosage to mild signs of toxicity. Since the leukemic cells are capable of developing resistance to the 4-amino antagonists of folic acid, no matter how brilliant the remissions are at first, they are, as with all other agents used in the treatment of acute leukemia, only temporary.

Development of Resistance

By methods somewhat similar to those used in bacteriology in developing strains of bacteria resistant to antibiotics, resistance to the chemotherapeutic effects of the anti-folics has been developed in cells of mouse leukemia,[85,86] and in *S. faecalis*,[51,87] *L. citrovorum*,[88] *L. arabinosus*,[27] and *E. coli*.[89] Studies by Hutchison *et al.* on the growth requirements of the Methotrexate-resistant mutant, *S. faecalis*/A, as contrasted to the parent Methotrexate-sensitive strain, show that it has developed a definite nutritional requirement for purines, and that in the presence of purines it has one-third the requirement of folic acid, but that in the absence of purines it requires 10 times the usual amount of folic acid.[90] Thus it appears that in gaining resistance to Methotrexate, *S. faecalis*/A has become more dependent on preformed purines and less on the *de novo* synthesis of purines for nucleic acid synthesis. Balis and Hutchison[91] found that *S. faecalis*/A incorporated significantly more labeled xanthine into RNA adenine and guanine than did the parent strain of *S. faecalis* when both were grown on a medium containing PGA. Broquist *et al.*[6] have demonstrated that *S. faecalis*/A can synthesize 80 to 200 times as much CF from a given amount of PGA as the parent strain, and Hutchison has shown that this conversion of PGA to CF in *S. faecalis*/A is much more resistant to concentrations of Methotrexate than in the parent strain.[90] 2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methylpyrimidine is 30 times as active as Methotrexate in preventing this conversion in *S. faecalis*/A.[90] Nichol has confirmed this observation on Methotrexate in the intact cells of *S. faecalis*/A,[7] but has also shown that an extract of sonically disintegrated *S. faecalis*/A cells, while still very effective in converting PGA to CF, is inhibited by much lower concentrations of Methotrexate.[92] He has postulated that part of the resistance to Methotrexate displayed by *S. faecalis*/A may be due to a decrease in permeability. Johnson, Skipper, and Hutchison, however, using labeled Aminopterin, noted that activity was found in equal quantities in both the sensitive and resistant cells of this mutant.[93]

Skipper *et al.*,[94–96] in a series of experiments on normal mice and mice with Methotrexate-sensitive and resistant strains of transplanted leukemias Ak4 and L1210, have shown that the incorporation of formate into the nucleic acid purines and pyrimidines is markedly reduced by prior treatment of the animal with Methotrexate. This occurs both in the viscera of the normal animal and in the viscera of the animal with a leukemic tumor, and in the leukemic tumor and leukemic spleen Using a strain of leukemia Ak4 resistant to Methotrexate, the incorporation of formate was much less inhibited by Methotrexate than in the sensitive strain,[95] and using a Methotrexate-dependent strain of L1210 leukemia, there was actually better incorporation of formate into the leukemic tumor after treatment with this drug.[96] Simultaneous studies showed, however, that Methotrexate had a severely inhibitory effect on the incorporation of formate in the viscera of these same animals. In the same strain of sensitive leukemia, Williams *et al.*[97] have shown that Methotrexate does not affect labeled PO_4 uptake into leukemic tumors.

Cross Resistance

Of primary interest to the clinician who is using the folic acid antagonists as chemotherapeutic agents would be studies on cross resistance and collateral sensitivity both in bacteria and in mouse leukemia. In bacteria, resistance to one 4-amino derivative of folic acid confers resistance to all, with cross resistance to the 4-hydroxy antagonists of folic acid as well.[27] With Methotrexate-resistant *S. faecalis* and *Leuconostic citrovorum*, there is also an increase of resistance to the 2,4-diaminopyrimidines, although this is less marked than with the 4-amino antagonists of folic acid.[90] Strains of these bacteria resistant to the diamino dichlorophenylpyrimidines are also more resistant to Methotrexate.

It is of interest, however, that the Methotrexate-resistant *S. faecalis*/A and *L. arabinosus*/A are more sensitive to mercaptopurine.[98] Similarly, Elion[99] has shown that a mutant of *L. casei* resistant to mercaptopurine is more sensitive to Methotrexate. Similar collateral sensitivity has been noted[100] in an Amicetin-resistant variant of *L. arabinosus*, which shows a five-fold increase in sensitivity to Methotrexate. It was also demonstrated that Methotrexate-resistant variants of *L. arabinosus*, *L. citrovorum*, and *S. faecalis* are more sensitive to Amicetin.

In mouse leukemia, there is cross resistance between the Methotrexate-resistant strains and all 4-amino antagonists of folic acid,[85,86] but there is no cross resistance to mercaptopurine,[101] Azaserine,[102] 8-azaguanine, or alpha peltatin.[103] In one Methotrexate-resistant line of mouse leukemia (I/A), some increase in sensitivity has actually been noted to mercaptopurine.[104] In strains of leukemia L1210 resistant to mercaptopurine, and other strains made resistant to or dependent on azaguanine, Law[105–108] has demonstrated an increased sensitivity to Methotrexate.

In children with acute leukemia, the development of resistance to Methotrexate confers cross resistance to all other folic acid antagonists, including the diamino dichlorophenyl pyrimidines,[46] but not to mercaptopurine or cortisone. Similarly, resistance to mercaptopurine or to cortisone is not necessarily accompanied by cross resistance to Methotrexate.[109]

Combination Therapy

The inability of any of these compounds to control mouse leukemia permanently has led to the suggestion that combination therapy with two or more agents which block at different levels of the same metabolic pathway might delay or prevent the development of resistance. Law[110] has demonstrated that Methotrexate and 8-azaguanine had a potentiating effect on leukemia L1210, and Skipper[111,112] has shown similar additive effects for Methotrexate and 8-azaguanine have a potensame strain of leukemia. Studies by Elion and Hitchings[113] have demonstrated that in bacteria anti-thymines in combination with anti-folic compounds have a marked synergistic inhibitory effect, and for that reason they suggested that the combination of Daraprim, 2,4-diamino-5 p chlorophenyl-6-ethylpyrimidine and azathymine might be of interest clinically. Azathymine alone or in combination with Methotrexate or Daraprim has not been shown to have synergistic effect against Sarcoma 180 or mouse leukemia. A preliminary trial of these combinations in patients has shown no beneficial effects, but further studies are in progress.

Clarke *et al.*[114] using Sarcoma 180 found that one quarter of the maximum tolerated doses of Azaserine and mercaptopurine in combination were more effective in inhibiting the tumor growth than full doses of either drug alone. These results have been confirmed by us in several strains of mouse leukemia, and patients have been under study with this combination for the past two years. Although the synergistic effects in children are not nearly so marked as in mice, it does appear that the addition of Azaserine to mercaptopurine at 2.5 mg/kg by mouth daily of each drug delays the development of resistance to mercaptopurine by from one to four months, and possibly also increases the incidence of remissions.[115]

Discussion

The synthesis and the screening of new compounds as potential antagonists of nucleic acid

precursors and the study of their effects in combination with the folic acid antagonists would appear to be a productive approach to the chemotherapy of the leukemias. It is also of considerable interest that, so far, among the various classes of folic acid antagonists, only the 4-amino PGA's seem to have a specific action on the acute leukemias of childhood, in that they will produce remissions without concomitant toxicity. It appears that exhaustive comparative studies of the mechanisms of action of these different groups in bacteria, in protozoa, and in normal and neoplastic tissues would be rewarding.

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Purine and Pyrimidine Antagonists

By GEORGE H. HITCHINGS, PH.D.

A MAJOR proportion of earlier investigations which dealt with the effects of antimetabolites *in vivo* were concerned with antivitamins. In many of these the symptoms of deprivation of the metabolite had been established by feeding experiments, and when a similar syndrome was produced by the antimetabolite it was reasonable to conclude that the substance possessed the mode of action *in vivo* which had been inferred from structural considerations and *in vitro* studies. The accumulation of a rather large volume of such information supports the view that *in vitro* studies provide presumptive definitions of mechanisms of action which may be extended usefully to other biological systems. However, when one is dealing with metabolites which normally are synthesized by the organism, the deficiency state is not available for comparison with the results of administration of the antimetabolite, and one can only infer what the consequences of deprivation of the metabolite might be from a general knowledge of the biochemical reactions in which it is involved. Consequently, much more in the way of experimental evidence is required to establish the mechanism of action in these instances than in those which involve known signs of deprivation. The reversal study (antagonism between metabolite and antimetabolite) is an important source of such evidence, but has certain pitfalls as, for example, the failure of folic acid to prevent the effects of its 4-amino analogue, aminopterin, in birds and mammals in contrast to the competitive effect in microorganisms. Metabolic studies and, finally, the study of analogues in combination may provide evidence of the mechanisms of action which may be quite convincing. The present paper deals primarily with some examples of the last two types of experiment and their applications to problems of the mechanism of analogue action.

From the Wellcome Research Laboratories, Tuckahoe, N. Y.

EFFECTS OF ANALOGUES

An extensive study of the effects of analogues of the natural purines and pyrimidines on the developing tadpole of *Rana pipiens*[1,2] was designed to glean information both as to the role of nucleic acid biochemistry in embryogenesis and the mechanisms of action of the analogues. A considerable number of inhibitory substances was found. When the dose response relationship was investigated, some tendency for higher concentrations to result in earlier inhibition was noted, but only within definite limitations, and it soon became apparent that whereas certain purine analogues were capable of producing an inhibition of very early development (stage 8, blastula) the antifolic acids were capable of inhibition no earlier than neurula (stage 12–13) at any reasonable concentrations (Table I). These facts suggested that early embryogenesis might occur at the expense of preformed purine-containing nucleic acid fragments which were stored in the ovum and that *de novo* synthesis (involving folic acid) might begin only at neurulation. Several facts support this view. The presence of very large stores of desoxypentose derivatives in the mature oöcyte (sufficient for the chromosomal DNA of about 10^5 somatic cells) has been confirmed.[3] These stores also are reduced when the female frog is starved during oögenesis (Table II) and

TABLE I

Inhibition of Development

Substance	Earliest Stage Inhibited
8-Azaadenine	8
6-Mercaptopurine	8
Pyrimethamine	14
Aminopterin	13

the resultant embryos are hypersensitive to the action of 6-mercaptopurine and other purine analogues.[4] Finally, added confirmation is being obtained from studies of the incorporation of radioactive formate, which is found to begin only at neurulation where

TABLE II

μg Desoxypentose per Oöcyte

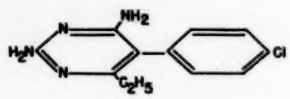
Diameter	Normal Females Cold TCA	Hot TCA	Starved Females Cold TCA	Hot TCA
1.6 mm	0.32	0.12	0.25	0.07
1.8 mm	1.00	0.22	0.45	0.09

DNA† of Mature Sperm = 3.6×10^4 μg

* TCA—trichloracetic acid.
† DNA-desoxyribonucleic acid.

the antagonists of the formylating coenzyme, folic acid, first become active, and to reach a high level at about the point that the stores of preformed nucleic acid fragments are approaching exhaustion. In this example, therefore, the acceptance at face value of the mechanism of action for 6-mercaptopurine which had been assigned on the basis of structural considerations[5,6] and microbiological studies[7,8,9] led, in an *in vivo* system, to a number of metabolic studies, the results of which are entirely consistent with the postulated mechanism of action.

The reversal experiment should not be neglected entirely as a means of demonstration



PYRIMETHAMINE
(DARAPRIM)
COMPOUND 50-63

Figure 1.

of the mechanism of action of an analogue. It may be pertinent here to recall the role of 2,4-diaminopyrimidines as antagonists of folic acid.[10,11] Since these substances are rather more remotely related to folic acid than are 4-amino folic acid (aminopterin) and similar structural analogues, it was of interest to determine whether folic acid antagonism could be demonstrated *in vivo* as well as *in vitro*. For this purpose young rats were fed a purified diet, otherwise adequate but lacking in folic acid, to which 1 g of pyrimethamine[12] (Daraprim; Fig. 1) per kg of diet had been added.

TABLE III

Effects of Metabolites on Toxicity of Pyrimethamine

Supplement	Amount per kilo diet	Average weight gain	No. of animals	No. of survivors
None	—	15	18	3
Folic acid	50 mg	9	6	0
Folinic acid	3.3 mg	43	5	4
Folinic acid	10 mg	44	13	10
Folinic acid	33 mg	82	5	5
Folic acid +	10 mg	9	7	3
Ascorbic acid	5 g			
Liver powder	50 g	101	24	24

This diet inhibited growth and was lethal within a few weeks; the animals showed, too, a typical folic acid deficiency syndrome. When the drug-containing diet was supplemented with folic acid, there was no effect; however, Leucovorin (folinic acid) was able completely to prevent death of the animals (Table III). Thus the effects of the diaminopyrimidines resemble closely those of aminopterin and A-methopterin *in vivo*. Perhaps one reservation should be made. It is not possible to titrate the pyrimidine against folinic acid *in vivo* over so wide a range of concentration as is possible with, for example, A-methopterin.[13,14] It is also apparent that liver powder is more effective in the prevention of the toxicity of pyrimethamine than is accounted for on the basis of its content of folinic acid (Table III, 50 g liver powder contain about 1.5 mg of folic acid + folinic acid). Nevertheless, the most probable explanation of these results, at present, would appear to be that the metabolite with which the pyrimidine competes is closely related to but not identical with folinic acid. Final confirmation of this view will have to await the isolation and testing of such a factor. In any case it is quite clear that the mechanism of action of the diaminopyrimidine *in vivo* is closely related to that demonstrated *in vitro*.

Folic Acid Antagonists

Experience with the folic acid antagonists may serve to illustrate one of the difficulties

in the way of a facile interpretation of the *in vivo* reversal experiment. It will be recalled that the early microbiological studies[15] had shown the action of aminopterin to be preventable by folic acid, although the two were not completely competitive. *In vivo*, however, folic acid had scarcely any effect on the toxicity of aminopterin, and it was only several years later after the discovery and synthesis of folinic acid that the role of aminopterin as a competitor of folinic acid, rather than folic acid, was appreciated. In this instance, therefore, the reversal experiment *in vivo* was at first somewhat misleading, and it seems probable that similar problems will arise with other analogues. Thus, for example, in microbiological experiments, the toxicity of 6-mercaptopurine is prevented by any of the four free purine bases,[7,8,9] but these are not effective *in vivo*.[16,17] However, several of the more complex purine-containing metabolites do effectively block the toxicity.[17] Thus, in a sense, this problem resembles that with aminopterin, in that the antidotal metabolite is more complex than the antimetabolite.

One of the more reassuring lines of evidence regarding the mechanisms of action of antimetabolites *in vitro* has been the ability to make predictions on the basis of *in vitro* studies and biochemical knowledge of the effects of combinations of analogues.

The chief biochemical pathway with which the purines and pyrimidines are concerned may conveniently be diagrammed as shown in Figure 2.

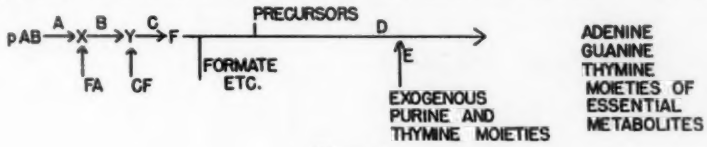


Figure 2.

In this scheme X represents the cellular equivalent of folic acid (FA) which may be formed *de novo* via *para*-aminobenzoic acid or by the incorporation of preformed folic acid, and Y represents the intracellular form of the citrovorum factor. Some member of this series of vitamins (F) is regarded as the formylating coenzyme which is involved in the incorporation of one-carbon fragments into suitable precursors to complete the purine and thymine moieties found in the nucleic acids and various nucleotides. When exogenous purines and thymine are available, they are incorporated (E) and join the main stream of *de novo* synthesis at some as yet unknown points. The availability of sulfonamides, competing in reaction A, diaminopyrimidines and other antifolic acids blocking reaction B, and of end product analogues competing at some point with the products of formylation allows one to test the effects of multiple blockade of a fundamental biochemical mechanism.

Combinations of sulfonamides with diaminopyrimidines provide systems in which the mechanism of action of the latter can be tested. Such combinations provide sequential[18,19] blockade of a biosynthetic pathway and would be expected therefore, to produce potentiative effects. In fact, a wide variety of combinations of sulfonamides with diaminopyrimidines and condensed systems containing the diaminopyrimidine moiety, has been tested *in vitro* and *in vivo* and potentiation has been observed in every instance.[20,21,22,23]

In Figure 3, the data are presented of an *in vitro* experiment using a combination of 2,4-diamino-5-(3′4′-dimethoxybenzyl) pyrimidine (compound 49-210) and sulfadiazine in the growth of *Proteus vulgaris*. In this graph, unity on each axis represents the amount (inhibitory concentration) of the substance required to reduce the growth of the organism to half-maximal. By the use of sub-inhibitory concentrations of one substance and graded amounts of the second, the composition of combinations which produce 50 per cent inhibition are determined graphically and are plotted as decimal fractions of the inhibitory concentrations. Where additive effects occur in combinations, the points fall on the line connecting the inhibitory concentrations of the

individual compounds.[19] Deviation of the locus of points to the left of this line signifies potentiation, and a quantitative expression of the degree of potentiation is found in the sum of the fractional inhibitory concentrations at

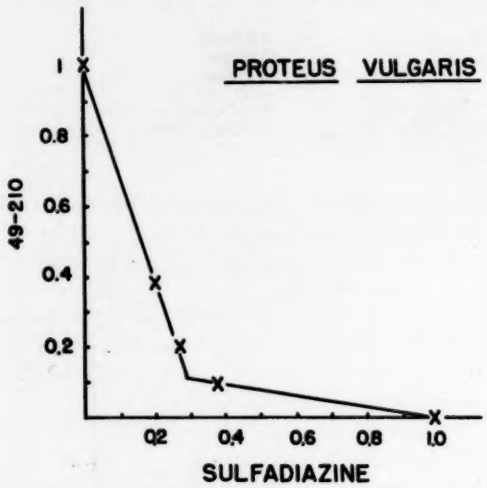


Fig. 3. Effects of combinations of a diaminopyrimidine and sulfadiazine on the growth of *Proteus*. Each point represents the composition of the additive which produces a 50% inhibition of growth. Unity on the ordinate is 30 μg of Compound 49-210 (2,4-diamino-5-(3',4'-dimethoxybenzyl)pyrimidine). Unity on the abscissa is 20 μg of sulfadiazine.

the intercept of the two lines of the figure. In Figure 3, this amounts to about 0.28 for sulfadiazine, and 0.12 for compound 49-210, a sum of 0.4. Thus the use of two analogues in combination has reduced the required quantity of inhibitor by 60 per cent.

Combinations

Since many of the diaminopyrimidines have chemotherapeutic activity, it is not surprising that a number of combinations of these drugs with sulfonamides have found chemotherapeutic applications. One of the more striking of these is the work of Eyles and Coleman[20] on *Toxoplasma gondii*. In previous work these authors had found sulfonamides to have some influence on the course of toxoplasmosis in mice, and pyrimethamine was somewhat more active, but neither of these drugs alone was capable of curing the infection. When they were used in combination, a strong potentiation occurred. In the most effective combination (Fig. 4) one twenty-fifth of an inhibitory dose of pyrimethamine together with one

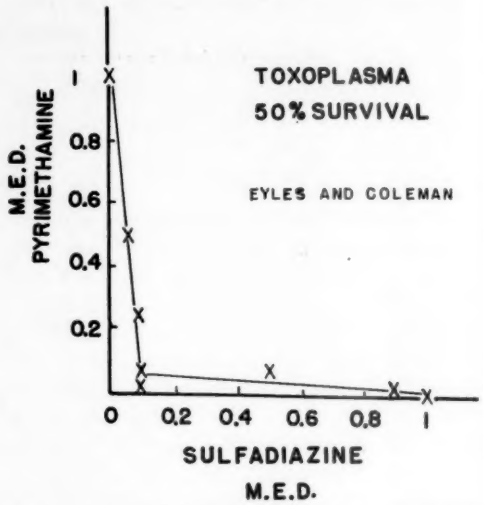


Fig. 4. Effects of combinations of sulfadiazine and pyrimethamine on survival of mice infected with *Toxoplasma gondii*. Drawn from the data of Eyles and Coleman.[20]

twelfth of an inhibitory dose of sulfadiazine was as effective in prolonging the survival of infected mice as a full inhibitory dose of either compound alone. Much more important, however, was the finding that, with combinations of the two drugs, effective clearance of the parasite (cures) could be obtained. Thus the improved potency obtained through the potentiative effect of the sequential blockade resulted in a qualitative improvement in therapy. These observations of Eyles have found a dramatic application in the cure of a case of acute toxoplasmosis which was the result of a laboratory infection in Eyles' own laboratory[24] and have stimulated clinical trials in toxoplasmic chorioretinitis with an apparently favorable outcome.

Finally, the mechanism of action of 6-mercaptopurine may be considered further in relation to its activities against neoplastic disease.[25] The reversal studies of Goldin[17] mentioned above are consistent with the view

that interference with purine metabolism is the primary *modus operandi* of this substance *in vivo* as well as *in vitro*. Potentiative effects with combinations of antifolic acids and 6-mercaptopurine have been observed *in vitro*[19] and *in vivo* with L 1210 leukemia.[26] These

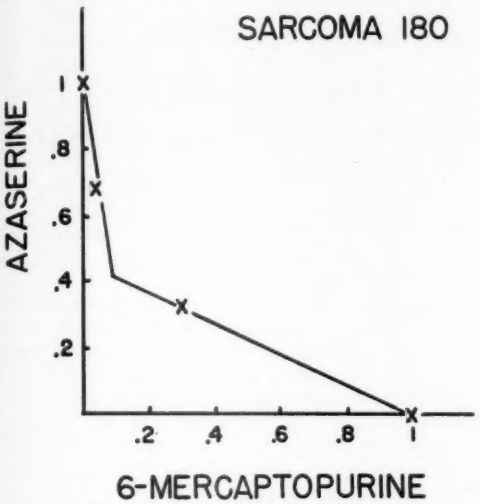


Fig. 5. Effects of combinations of Azaserine and 6-mercaptopurine on the growth of sarcoma 180 in mice. Unity on the ordinate is 7.5 mg/kg of Azaserine; on the abscissa, 155 mg/kg of 6-mercaptopurine.

are regarded as the result of concurrent blockade, in the scheme above, of the main *de novo* synthetic pathway at B, and the by-pass mechanism at E. The observations of Clarke[27] dealing with the potentiation of 6-mercaptopurine by Azaserine in the inhibition of sarcoma 180 are of considerable interest. In Figure 5 the data of Clarke have been plotted in terms of fractional inhibitory concentrations.* It is apparent that potentiation does occur and that a combination of 0.4 of an inhibitory dose of Azaserine and 0.1 of an inhibitory dose of 6-mercaptopurine has the effect of a full inhibitory dose of either drug alone. Moreover, as in other instances, it was possible to attain greater inhibitory effects and a greater percentage of tumor regressions with combination therapy than with either substance given singly (Table IV).

When this was first discovered, the biochemical interrelationship between the two inhibitors was not apparent, but this has been

TABLE IV**

Effects of Combinations of Azaserine and 6-Mercaptopurine on Recoveries from Sarcoma 180

Exp. No.	Compound	Dose mg/kg	Recoveries*
1	6-Mercaptopurine	50	2
2	Azaserine	10	3
3	6-Mercaptopurine +	25	8
	Azaserine	5	
4	6-Mercaptopurine +	12.5	9
	Azaserine	2.5	
5	Controls	—	0

* 19 mice in Exp. No. 2, 20 mice in each of the others.

** Abstracted from the data of Clarke *et al.*[27] (To be published in full).

clarified by recent observations of Skipper[28] and Buchanan,[29] who have shown that Azaserine interferes with the formylation specifically of 4-amino-imidazole-5-carboxamide ribotide, a precursor of inosinic acid (hypoxanthine-ribosephosphoric acid). 6-Mercaptopurine also appears to become involved rather specifically with the metabolism of a hypoxanthine-containing metabolite.[8] Figure 6 may be regarded as a schematic representa-

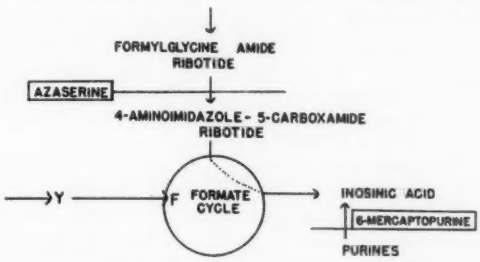


Fig. 6. Diagrammatic representation of biochemical reactions which involve Azaserine and 6-mercaptopurine.

tion of the interrelationship between the two blocking agents, and an enlargement of a segment of the general scheme which was diagrammed above. The synergism between Azaserine and 6-mercaptopurine would thus be

* These data were drawn from experiments not primarily designed for this purpose, and some extrapolation of the dose-response curves for the individual drugs was necessary. However, the validity of this procedure is supported by collateral experiments with the same compounds.

viewed as an additional example of the concurrent blockade of two pathways to the same essential metabolite.

Comment

Studies *in vivo* of a considerable number and variety of antimetabolites have, with reassuring frequency, given results which are consistent with the mechanisms of action which have been assigned on the basis of structural considerations and *in vitro* studies. Such assurance is of particular importance in the application of antimetabolites to practical problems of chemotherapy. It has led, with increasing frequency, to the use of combinations of antimetabolites which have resulted in enhanced therapeutic effects. At the same time, potentiation in drug combinations can be viewed as an integration of the antimetabolic activities of the individual drugs, and can contribute supporting evidence regarding the mechanisms of action of the substances involved. That potentiative effects have become in a measure predictable offers considerable encouragement to the biochemical approach to chemotherapy.

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Anti-Vitamin E Stress Factors as Related to Lipid Peroxides

by EDWIN L. HOVE, PH.D.

IN THE study of vitamin E metabolism, the expression "stress factor" has gained wide usage. This term is applied to any substance that precipitates a pathological condition curable or preventable by vitamin E. At times the term has included deficiencies of other dietary nutrients, such that the animal has a more urgent need for vitamin E.

To consider a stress factor as an antimetabolite may seem an error to some, since there is no obvious relation to the ideas of "structural analogues" or "competitive inhibitions." However, restriction of the discussion to such terms would suggest a knowledge not actually possessed as to the function of vitamin E: namely, that it acts as a coenzyme. On the other hand, there is substantial evidence of a non-enzymatic role of vitamin E in animal metabolism.

VITAMIN E AS AN IN-VIVO ANTIOXIDANT

The concept that vitamin E may act in the body as an antioxidant has been reviewed by Mason[1] and Dam.[2] Fundamental to this theory was the demonstration by Dam[3] that lipid peroxides occurred in measurable amounts in body lipids of vitamin E-deficient animals.

The postulated metabolic scheme for vitamin E function is outlined in Figure 1. Tissue unsaturated fat, oxidized by metal-porphyrin catalysts such as myoglobin or hemoglobin[4] pass through various intermediate peroxides to the relatively stable hydroperoxide. This is further disrupted catalytically to active oxygen and other rupture products. Dubouloz and co-workers[5] isolated a heme-pigment from liver that catalyzed the breakdown of lipid peroxides. Both of the heme-catalyzed steps are capable of being inhibited by vitamin E,[6-9] at least *in vitro*.

The lipid peroxides formed by this reaction are assumed to produce the multiple disturbances observed in animals deprived of vitamin E. Perhaps the lipid peroxides act directly on capillary walls or tissues; or, more likely, the oxidation of sulfhydryl groups by the lipid peroxide, as shown by Dubouloz and Fondarai,[10] disrupts various enzyme systems. Variations in the pathological picture in the vitamin E-deficient state are assumed to reflect variations in the particular sets of enzyme systems which may be most susceptible to the lipid peroxide, due either to species differences or to dietary limitations of one or another essential nutrient.

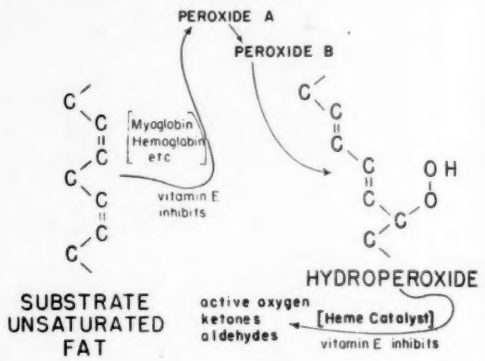

Fig. 1. A postulated metabolic role for vitamin E as an *in vivo* antioxidant.

If the role of vitamin E as an *in vivo* antioxidant is granted, then destruction of vitamin E during peroxidation represents the normal pathway and function, and not an artifact.

VITAMIN E ANTIMETABOLITES

Vitamin E antagonists may be considered to be substances that promote the reaction

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outlined in Figure 1. Obviously, an increase in the substrate unsaturated fat would do this. Several materials not normal to animal tissue act catalytically in this system. Examples of these are the esters of o-cresol, carbon tetrachloride, pyridine, sulfaguanidine, sulfathiazole, and sodium sulfite. In each of these examples vitamin E provides a measure of protection against toxic manifestations in the animal body.

Reliance on pathologic similarity as an indication of vitamin E antagonism may be misleading. For example, fetal resorption is a classical symptom of the vitamin E-deficient state in rats or mice, yet resorption has been produced by rancid lard,[11] α-tocopheryl quinone,[12] high levels of vitamin A,[13] a deficiency of essential fatty acids,[14] or by experimental lathyrism.[15] Against none of these was vitamin E of benefit. Some benefit against the paralysis of lathyrism was noted by Lee,[16] and the similarity of symptoms was pointed out by Ponseti and Shepard.[17]

Substrate Unsaturated Fat

The most widely known vitamin E antagonist is cod liver oil. Its relation to muscular dystrophy in herbivora has been reviewed by Mackenzie[18] and by Blaxter.[19] According to Dam,[2] all vitamin E deficiency symptoms in all animals are exaggerated by cod liver oil supplements. However, there is nothing specific about the action of this oil. Corn oil or soybean oil, when stripped of their tocopherol, were as effective as cod liver oil in inducing muscular dystrophy in rabbits.[20] Sherman, in our laboratory, clearly demonstrated in 1941[21] that the so-called sparing effect of vitamin E on carotene occurred only in the presence of linoleic or linolenic acid. Sunflower seed oil, as well as high levels of linoleate, have been shown[22] to produce paralysis and death in rats, preventable by vitamin E. Filer, *et al.*[23] obtained similar results with linseed oil fatty acids. Numerous other reports occur in the literature, all showing that unsaturated fats precipitate vitamin E deficiency symptoms, which, if not too severe, could be corrected by additional vitamin E supplements.

The stress effects of many natural oils are masked because they contain vitamin E. We recently pointed out the close correlation between the levels of linoleic acid and total tocopherols in most fats.[24] Earlier we had shown that essential fatty acid deficiency in the rat was aggravated by vitamin E.[25]

Unsaturated fat does increase the vitamin E requirement of animals. Unfortunately, however, little quantitative work has been done on this relation, and it is not yet possible to plot the unsaturation-potential of the diet against the vitamin E needs of any animal. If such a curve were available, it might, by extrapolation to zero stress factor, indicate whether a vitamin E requirement existed at all in the hypothetical absence of essential unsaturated fat. Singsen and co-workers[26] have made a beginning in the quantitative approach in their study on the relation of fish oils to encephalomalacia in chicks.

An example of the cod liver oil-vitamin E antagonism from more recent reports has to do with the production of liver necrosis in swine. This important vitamin E deficiency syndrome was first described in the rat by Schwartz.[27]

Fatal liver necrosis was shown by Obel[28] to occur in swine fed a diet of yeast, minerals, carbohydrate, and 6 per cent cod liver oil. Oral supplements of vitamin E gave protection. However, when the cod liver oil was raised to 12 per cent, the same level of vitamin E no longer protected; the hogs died with typical vitamin E deficiency syndrome. We have produced a similar condition when only 2 per cent cod liver oil was used in a somewhat different basal diet. The type of liver damage is shown in Figure 2. Half of the pigs on the diet without vitamin E developed the fatal acute hemorrhagic necrosis of the liver, while the survivors all had postnecrotic scarring. The composition of the muscle fat of these pigs was altered, as shown in Table I. The total fat increased; it was yellowish and had a demonstrable linolenic acid level. On the other hand, the essential fatty acids, such as arachidonic and linoleic, decreased on the per cent-of-fat basis. Oxidation of the fats was indicated by the sharp rise in conjugated

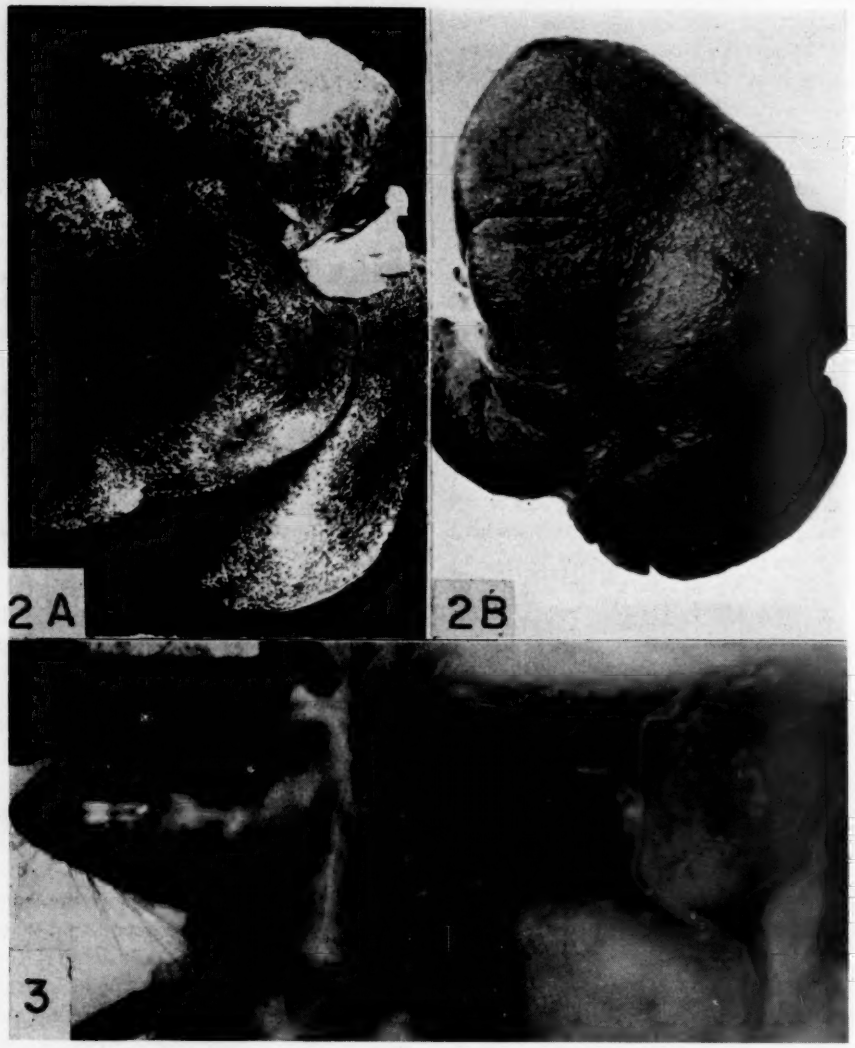

Fig. 2-A. Cod liver oil-induced fatal liver necrosis in vitamin E-deficient swine. 2-B. Cod liver oil-induced chronic liver damage in vitamin E-deficient swine.

Fig. 3. Gross dissection of a tri-o-cresyl phosphate-poisoned rat, showing liver necrosis and lung hemorrhage.

TABLE I

Altered Fat Composition in Vitamin E-Deficient Swine[1]

(2% CLO in ration of SBOM,[2] Minerals, CHO, and Lard)

	Vitamin E-deficient	Added vitamin E
Incidence liver damage	6/6	0/5
Muscle fat, % wet	6.3	3.8
Iodine number	79.1	79.6
Linoleic acid, % of fat	3.11	5.36
Linolenic acid, % of fat	1.73	0
Arachidonic acid, % of fat	1.36	2.19
Pentaenoic acid, % of fat	0.72	1.14
Conj. dienes liver fat	1.49	0.71
Lipid peroxide, mEq/kg	13	3

[1] Abstracted from Hove and Seibold, *J. Nutrition* (in press).

[2] Soy bean meal.

dienes, especially in the liver fat, and by a rise in lipid peroxide in muscle fat.

This swine disease occurs naturally to an alarming degree in Sweden, according to Obel. It is not known to occur in this country. However, a strikingly similar pathology has been seen in a fatal condition called "moldy corn disease" occurring in several Southern states during drought conditions.[29]

Esters of o-Cresol

The next vitamin E antagonist to consider is tri-o-cresyl phosphate (TOCP). This compound has had wide industrial use as a plasticizer, a solvent in furniture polishes, and a solvent in the preparation of modified fibers for clothing; recently it has gained a certain usage as a gasoline additive. Cresols and their esters occur naturally in many plants, especially of the woody variety.

Paralysis in humans has frequently occurred due to cresyl-phosphate poisoning. This was first reported by Smith *et al.*[30] in 1930, who described a condition of "Ginger Paralysis" that occurred in hundreds of people about a week after drinking a certain alcoholic extract of Jamaica ginger root. The paralysis was produced experimentally in rabbits, chickens, and calves with an approximate dose of 0.1 g tri-o-cresyl phosphate (TOCP) per kg body weight. The paraisomer and the free cresol were said not to be paralytic.

TABLE II

Effect of Tri-O-Cresyl Phosphate (TOCP) on Incidence and Time of Development of Fatal Liver Necrosis in Rats

Casein in diet	With TOCP, 0.1%			Without TOCP		
	No vit. E		Plus vit. E	No vit. E		Plus vit. E
	%	*wk*	%	%	*wk*	%
10%	100	3.3	0	50	7.8	0
20%	33	6.8	0	0	—	0

The similarity between Ginger Paralysis and vitamin E deficiency was suggested by Bloch,[31] who later[32] prevented the creatinurea of cresyl-phosphate-poisoned rabbits with high doses of vitamin E. The antagonism between the cresyl phosphate and vitamin E was confirmed by several laboratories.[33,34,35] However, it has also been shown that the succinate[34,36] or the acetate[36] of o-cresol are equally as antagonistic to vitamin E as the phosphate.

A rat poisoned with TOCP had the appearance shown in Figure 3. Necrosis of the liver, with hemorrhage in the lungs, and rosy-hued flesh are evident. The data in Table II show that the fatal pathological changes can be prevented by vitamin E. It will be noted from this data that the cresyl phosphate increased the incidence and decreased the time of occurrence of fatalities in rats, not only on the 10 per cent casein diet, but also on the 20 per cent casein diet. Vitamin E gave complete protection at both levels.

The unsaturated fat of the animal body is affected by TOCP. Extensive demyelination of peripheral and spinal cord nerve occurs, according to Barnes and Denz[37] and previous workers. It was also noted, as shown in Table III, that carcass fat decreased in amount and was altered in composition when TOCP was fed in the diet.[38] When vitamin E was not fed, the carcass fat was yellow and had a high linolenic acid content. The arachidonic acid content decreased sharply and consistently. Although these changes were not too evident in the simple vitamin E deficiency, it may be recalled that quite similar changes had been noted in hogs deficient in vitamin E. The conjugated diene increased, and this was reflected by higher lipid peroxide. The tocoph-

TABLE III

Effect of Tri-O-Cresyl Phosphate (TOCP) on Composition of Rat Carcass Fat[1]

	With TOCP, 0.1%		Without TOCP	
	No vit. E	Plus vit. E	No vit. E	Plus vit. E
Total fat, % wet wt	7.52	13.16	13.24	15.20
Linoleic acid	6.64	7.76	8.02	8.17
Linolenic acid	0.17	0	0	0
Arachidonic acid	0.70	1.25	1.30	1.47
Conj. diene	1.08	0.24	0.22	0.18
Tocopherol, mg/g fat	0.007	0.04	0.02	0.07

[1] Abstracted from Ref. 33.

erol content decreased whenever TOCP was administered.

The evidence indicates that o-cresyl phosphate caused an oxidative state in body lipids, which could be prevented by vitamin E; but other effects occurred on which vitamin E had no influence, such as depressed growth rate.[38,39]

Carbon Tetrachloride

Under rather limited conditions of diet and dosage, vitamin E supplements protected rats against death[40] and liver damage[41] caused by carbon tetrachloride, another vitamin E antagonist. Typical results on survival time curves obtained with supplements of vitamin E are shown in Figure 4. The diet contained

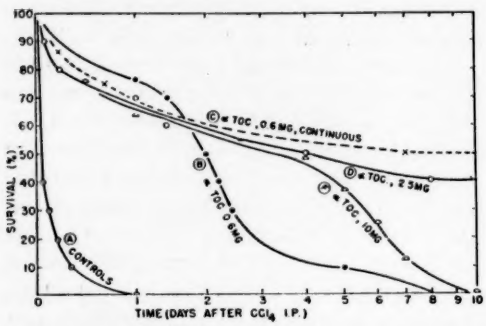

Fig. 4. The effect of vitamin E at different levels in protecting rats on a 10% casein diet against death after injections of single doses of carbon tetrachloride (1 ml/kg).[40]

10 per cent of casein but did not contain cod liver oil. Under these conditions, spontaneous deaths, with gross liver damage in uninjected rats, did not occur. The data in this graph indicate that in rats without vitamin E death resulted very quickly after injection of 2 ml of CCl_4 per kilogram of body weight. These results have been confirmed in part.[42,43]

TABLE IV

Creatine Metabolism as Related to CCl_4 and Vitamin E in Rats[1]

Supplements		Creatine of:		Creatine synthesis by liver slices from guanido-acetic and methionine
CCl_4 ml/wk	Vit. E mg/day	Muscle mg/g,dry	Urine mg/kg/day	μg/hr/g liver
0.05	0	13.0	61.4	30.3
0	0	15.4	21.3	77.6
0.05	1	16.5	8.9	70.3
0	1	17.8	5.4	112.0

[1] Abstracted from Ref. 39.

Carbon tetrachloride poisoning increased the endogenous oxygen uptake by liver homogenates,[44] and this could be largely prevented by vitamin E supplements to the intact animal. There is a possibility that the increased oxygen uptake in liver, as well as in muscle of vitamin E-deficient animals, is the result of catalytic peroxidation of unsaturated fat in the tissues, and not due to an increased rate of glucose respiration.

Creatine metabolism is disrupted by carbon tetrachloride.[45] The data in Table IV show a great increase in creatine excretion in the urine caused by chronic poisoning, and largely preventable by vitamin E. The muscle creatine was correspondingly low. A great decrease in the ability of liver slice to synthesis creatine from guanido acetic acid plus methionine was noted. The changes in creatine metabolism due to CCl_4 appeared to be exaggerations of the existing vitamin E deficiency, and partial protection against these changes was given by vitamin E.

Pyridine

The toxicity of pyridine parallels that of carbon tetrachloride in many ways, especially as related to dietary protective substances.[46] Table V shows the protective effect of vitamin E against pyridine given to rats on the 10 per cent casein diet. Without vitamin E, the total deaths were 64 per cent and liver hemorrhagic

TABLE V

Effect of Vitamin E on Pyridine Toxicity in Rats on 10% Casein Diet[1]

Method of pyridine administration	Added vitamin E %	Number of rats	Deaths no	Liver damage no
Dietary, 1%	0	32	18	21
	0.01	31	6	6
Injected, 0.5 g/kg	0	18	14	16
	0.01	13	1	3
Total	0	50	32 (64%)	37
	0.01	44	7 (16%)	9

[1] Abstracted from Ref. 40.

necrosis was 74 per cent. With vitamin E supplements prior to poisoning, the death loss was only 16 per cent and liver damage only 20 per cent. About a third of the animals that survived the pyridine developed severe muscular dystrophy.

"Sulfa Drugs"

The various "sulfa" drugs have been reported to be vitamin E antagonists. In 1940 it was reported[47] that sulfathiazole caused a syndrome in humans similar to amyotrophic lateral sclerosis. This was correctable and preventable by vitamin E supplements in the one case tried. Two years later Daft *et al*[48] had reported that both sulfathiazole and sulfaguanidine caused muscle lesions in rats, and that increased vitamin E in the diet prevented such lesions. Some difficulty was encountered in repeating the effect of sulfaguanidine as an anti-vitamin E factor, and in fact Dam[49] reported that sulfaguanidine could replace part of the vitamin E requirement of rats. Very recently[50] it was found that heart lesions in mice fed sulfaguanidine could be prevented by either vitamin E or vitamin K.

Sodium Sulfite

This compound has recently come to the fore as an anti-vitamin E factor. Irving *et al.*[51] noted that sodium sulfite in the diet caused symptoms of vitamin E deficiency, especially whitening of the teeth, and that this was preventable by increased levels of vitamin E. Miller *et al.*[52] added 0.1 per cent of sodium bisulfite to the chick diet and markedly increased the incidence of encephalomalacia.

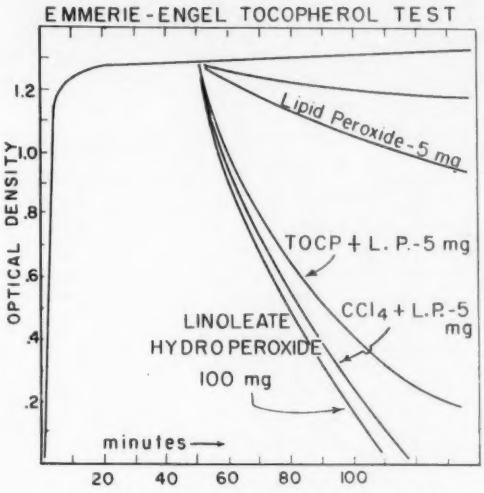

Fig. 5. Destruction of the red color of the Emmerie-Engel test for tocopherol (180 μg) in 10 ml ethanol, by carbon tetrachloride or tri-o-cresyl phosphate (TOCP) added at 500-mg levels with 5 mg of linoleate peroxide. (The unmarked line represents the effect of the solvents alone.)

Mode of Action of Vitamin E Antagonists

The mode of action of vitamin E antagonists was earlier suggested to be as pro-oxidants for unsaturated fatty acids, thereby increasing the need for vitamin E to inhibit this increased fatty peroxidation. Let us review the evidence that the several, very dissimilar vitamin E antagonists are, in fact, capable of promoting oxidative states in unsaturated lipids.

The encephalomalacia-promoting property of sodium bisulfite was shown to correlate with its ability to produce rancidity in dietary fat by Miller *et al.*[52] Sulfa drugs have not been studied in terms of oxidative-reductive status in lipids. Pyridine was shown to actively promote oxygen uptake by lard,[46] and to catalyze the lipid peroxide destruction of carotene.[38] CCl_4 and TOCP both catalyzed the peroxide destruction of carotene. Another example of the pro-oxidant activity of these solvents is shown in Figure 5. The Emmerie-Engel color test for tocopherol depends on the stoichiometric formation of ferrous ions, which then give a red color with dipyridyl. Large amounts of linoleate peroxide destroyed this color. With smaller amounts of the lipid per-

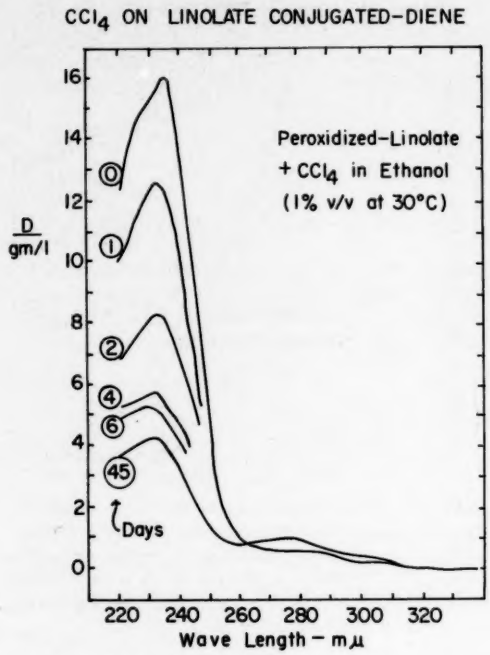

Fig. 6. Carbon tetrachloride-induced loss of conjugated diene by linoleate hydroperoxide (1% by volume in ethanol, with 1% CCl_4, and held at 30° C).

oxide, only a slight destruction of color was noted; however, when carbon tetrachloride or o-cresyl phosphate were combined with the small level of linoleate peroxide, the rate of color loss became rapid. These highly polar solvents may promote hydroperoxide disruption with liberation of "active" peroxides.

Disruption of the hydroperoxide of linoleate by CCl_4 is shown in Figure 6. Loss of the conjugated double bond gradually occurred when CCl_4 was stored at 30° C with linoleate hydroperoxide in alcohol. Details of this effect are given in Table VI. Conjugated dienes and peroxide number decreased when CCl_4 was present. However, vitamin E prevented this effect of CCl_4. The decrease in iodine number, conjugated dienes, and the increased density suggested that the carbon tetrachloride had added across double bonds in the conjugated diene. Kharasch *et al.*[53] note that CCl_4 adds across olefinic double bonds in the presence of organic peroxide, or other generator of free radicals. Since the linoleate hydroperoxide molecule contains a built-in organic peroxide, it seems reasonable to assume that the Kharasch addition reaction did occur in the absence of vitamin E.

TABLE VI

Carbon Tetrachloride-Catalyzed Decomposition of Linoleate Hydroperoxide (1% in Ethanol) After 30 Days at 30°C.

	Analysis of samples with additions of		
	None	CCl_4	CCl_4 1% α-tocopherol (1 mg)
Conj. diene, %	15.58	3.17	14.21
Linoleic acid, %	55.50	51.79	47.74
Linolenic acid, %	0.42	2.93	0.33
Arachidonic acid, %	0.31	0.82	0.27
Peroxide number, mEq/kg	3,530	705	2,860
Iodine number	136	95.3	109
Density, g/ml	0.873	0.925	0.861

Discussion

Probably there are many substances in the form of drugs or food constituents capable of being vitamin E antagonists. At present there are only suggestions, as outlined above. Certainly there is no present reason to believe that the anti-vitamin E pro-oxidant aspects of the several toxicities represent the major pathway of the drug action. Large doses of the compounds readily break through the protective barrier of vitamin E at any level, as might reasonably be anticipated. However, small doses over a long period of time might be expected to produce lipid peroxides in the tissues when insufficient vitamin E has been taken in. It is interesting that Dam *et al.*[54] noted lipid peroxides in human venous diseases.

Summary

The pathological states observed in vitamin E deficiencies have been suggested to result from the action of lipid peroxides on sulfhydryl-sensitive enzyme systems. Vitamin E antagonists, such as unsaturated fat, o-cresyl esters, carbon tetrachloride, pyridine, sulfathiazole, sulfaguanidine, and sodium sulfite, may act as pro-oxidants. They promote the production of lipid peroxides in the body tissues, and thus increase the need for vitamin E.

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Another paper presented at the Symposium on the Antimetabolites "Vitamin K and the 4-Hydroxycoumarin Anticoagulants," by Dr. Karl Paul Link, was not received in time for publication.

Editorial

The Kwashiorkor Syndrome

Publication of the monographs, *Kwashiorkor in Africa*[1] and *Síndrome Policarencial Infantil (Kwashiorkor) and its Prevention in Central America*[2] has focussed attention on a syndrome which is probably the most serious and widespread nutritional disorder in the world today. While this syndrome is undoubtedly rare in the United States, a few cases have been encountered.[3] The syndrome has been described in many countries since early in this century, under a variety of names, some descriptive of clinical findings, others suggestive of etiologic relationships.[2] The joint FAO/WHO Expert Committee on Nutrition used the simple term "protein malnutrition" at the conference in Gambia in 1952.[4]

Regardless of designation, the primary cause of kwashiorkor has been shown to be a diet deficient in good quality protein and the syndrome appears in the age group where protein need is greatest. Manifestations of the syndrome vary somewhat from one area of the world to another, due, in large part, to differences in diet. In many localities, caloric undernutrition and vitamin deficiency complicate deficiency of protein.

In Africa, the fundamental signs of kwashiorkor are considered to be retarded growth in the late breast-feeding, weaning, and post-weaning period, alterations in skin and hair pigmentation, edema, fatty infiltration, cellular necrosis or fibrosis of the liver, and a high mortality unless good dietary protein is provided.[1] Atrophy of pancreatic acini may also be a fundamental lesion.

In Central America, protein deficiency is accompanied, in most instances, by inadequate caloric intake and often by vitamin deficiency as well. Thus, caloric undernutrition exerts a more important role in the clinical picture of kwashiorkor in this hemisphere than in Africa.[2] Another difference between the two areas is the high incidence of the syndrome in children over four years of age in Central America. In both areas, important features of the disorder, in addition to the basic findings noted above, are a variety of dermatoses, gastrointestinal disturbances (anorexia, digestive upsets, diarrhea and mild steatorrhea), peevishness and mental apathy and anemia which may be normocytic or slightly macrocytic. The skin lesions are multiple in type: hyperpigmentation is common, with lesions which resemble pellagra in appearance but not in distribution; hyperkeratinization and vascular changes occur frequently. The etiology of these dermatoses is uncertain. Multiple deficiencies may be involved; an inadequate supply of vitamin A, riboflavin, niacin, or other vitamins has been suggested. The factors responsible for the changes in texture and color ("dyspigmentation") of the hair likewise have not been elucidated. Deficiencies of sulphur-containing amino acids or of vitamins, especially those of the B complex, have been considered but not proved. Basic information on the pathogenesis of the various aspects of the kwashiorkor syndrome may assist in delineating functions of individual amino acids and may uncover important nutrient interrelationships.

It is recognized that diarrhea, infectious diseases, and digestive upsets may act as secondary or precipitating causes of the kwashiorkor syndrome. The role of these factors needs to be clarified. Relationships

between protein malnutrition and parasitic infection and other diseases common in the tropics require elucidation.

Important aspects of the prevention and treatment of kwashiorkor remain to be solved. While therapy with skim milk powder is highly effective, this is practical only as an emergency measure. A search for inexpensive sources of good protein which may be used in child feeding is under way in many countries.[5] It is essential to determine whether a combination of vegetable proteins will prove a satisfactory substitute for animal protein. Even when satisfactory protein supplements become available, practical solution of the problem will be extremely difficult. Poverty, ignorance, food habits and prejudices, and an unhygienic environment are some of the co-existing factors requiring attention.

In areas where kwashiorkor is common, a high incidence of chronic liver disease is noted in adults, not only cirrhosis but also primary carcinoma. More accurate knowledge of the role of diet and individual nutrients in the development and therapy of these pathologic states is needed. Solution of the serious problems of protein deficiency should be a challenge to all those interested in the science of nutrition.

—GRACE A. GOLDSMITH, M.D.
Division of Nutrition and Metabolism, Dept. of Medicine, Tulane University, New Orleans, La.

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The Word "Kwashiorkor"

"As the earliest importer of the word 'kwashiorkor,' . . . I feel I must defend . . . its use.

"What is there absurd? This word means this disease. It is defined as 'the disease the child gets when the next baby is born.' And in most countries it has been found that this is indeed the situation often associated with its occurrence. It has now been fairly extensively investigated, and it is proved to be largely due to protein malnutrition. But possibly this is not the whole story. Perhaps certain amino-acids are more important than others. Perhaps certain minerals, toxic drugs, or herbs will prove to be part of the picture. Until we know more accurately what are the factors leading to this disease, some people prefer to use a word which conveys an idea without yet making unjustified claims to detailed knowledge.

"Some of my medical friends were horrified at the word 'kwashiorkor,' but now many of them have learnt to pronounce it quite intelligibly. I am sure they will enjoy learning to say 'tzipitl.'

"But beri-beri is still called beri-beri although we now know it is due to aneurine deficiency. Rickets (a word used probably among primitive Anglo-Saxon tribes) is still called rickets though we now recognise it as vitamin-D deficiency. 'Onyalai,' as far as I know, has no serious rivals. What about 'yaws' or even 'malaria'? 'Kwashiorkor' is to some extent a confession of ignorance. But I cannot see why, because it is African, it is therefore unacceptable. Scipio, at any rate, would not have been surprised."

—Cicely D. Williams (Letter to the Editor). *Lancet* 1: 1071, 1955.

Dietotherapy

THE SODIUM-RESTRICTED DIET

By CORINNE H. ROBINSON*

A RECENT publication prepared by a committee of the Food and Nutrition Board[1] presents a comprehensive report of the uses, planning, and administration of sodium-restricted diets. Such diets may be useful when they are planned to provide a sodium intake at a level adjusted to the individual's requirements and to include the essentials of normal nutrition.

NOMENCLATURE FOR SODIUM-RESTRICTED DIETS

Terms such as "salt-free," "low-salt," and "salt-poor" refer simply to the exclusion of table salt and perhaps to the salt used in cookery, and do not recognize the many sodium products other than sodium chloride which may be present in foods, either naturally or as a result of processing. Therefore, diets should be designated as "sodium-restricted."

Some conditions require a severe restriction of sodium to approximately 250 mg, while for other conditions sodium levels of 500 mg or 800 mg may prove to be satisfactory. Only by specifically designating a level of sodium in milligrams per day can the physician be assured that a satisfactory diet plan will be worked out by the dietitian or nutritionist. It should be clearly understood, however, that the data on the sodium content of foods are by no means as complete as might be desired, and that values for a given food may vary quite widely from those in published tables. Hence, a specific designation, such as 500 mg sodium diet, establishes a range for planning the diet but does not denote a quantitatively exact amount.

SODIUM CONTENT OF FOODS

The usual daily intake of sodium is about 3 to 6 g. When the diet is prepared without salt, the sodium level may be reduced to approximately 1000 to 1500 mg, depending upon the choice of foods, the amounts of animal foods used, and the amounts of salted bread and butter included.

In general, animal foods contain relatively important amounts of sodium. Therefore, milk, eggs, meat, poultry, and fish must be used in measured amounts. Organ meats and shellfish contain somewhat greater amounts of sodium, dark meat of poultry appears to be of higher sodium value than light meat, and egg white is considerably higher in its concentration of sodium than is egg yolk.

Fruits, cereals, and most vegetables are insignificant sources of sodium and may be used without restriction in the diet. Certain vegetables such as spinach, beet greens, celery, and a few others noted in the lists below should be omitted from the diet when sodium restriction is severe because their use would necessitate the exclusion of more important protein foods. The water supply[1] in certain communities may contribute significant amounts of sodium.

Table salt is the principal source of dietary sodium. It is used not only in the home preparation of foods but finds its way into many manufactured food products for flavor, as a preservative, as a means to separate peas or lima beans for quality before canning and freezing, and in a rinse to prevent the discoloration of fruits.

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TABLE I

Nutritive Value for a Basic Pattern for the Sodium-Restricted Diet[1]

Food	Household Measures	Weight	Energy	Protein	Fat	Carbohydrate	Minerals			Vitamins				
							Na[2]	Ca	Fe	A	Thiamine	Riboflavin	Niacin	Ascorbic acid
		g	*cal*	*g*	*g*	*g*	*mg*	*mg*	*mg*	*IU*	*mg*	*mg*	*mg*	*mg*
Milk	2 cups	488	335	17	19	24	244	576	0.4	780	0.18	0.84	0.6	6
Egg	1 medium	54	75	6	6	0	70	26	1.3	550	0.05	0.14	Tr.	0
Meat, poultry, or fish	5 ounces (raw weight)	120 (cooked)	365	28	27	0	104	10	3.5	2280[3]	0.30	0.40	6.9	0
Whole-grain or enriched cereal[4]	1/2 cup	30 (dry)	110	3	Tr.	24	Tr.	12	0.9	0	0.16	0.05	1.0	0
Whole-grain or enriched bread	4 slices	100	275	9	3	52	30	79	1.8	0	0.24	0.15	2.2	0
Potato	1 medium	150	125	3	Tr.	29	4	17	1.0	30	0.14	0.05	1.5	21
Leafy, green or yellow vegetable[5]	1–2 servings	150	45	3	Tr.	9	14	67	1.5	1320	0.12	0.11	1.0	39
Other vegetables[6]	1 serving	100	35	1	Tr.	8	4	19	0.6	770	0.06	0.06	0.7	17
Citrus fruit	1 serving	100	45	1	Tr.	12	1	27	0.4	120	0.07	0.03	0.2	47
Other fruit[7]	2 servings	200	125	1	1	32	5	24	1.0	1200	0.08	0.08	0.8	18
Butter or fortified margarine	2 tablespoons	30	215	—	24	Tr.	3	Tr.	0	990	Tr.	0	Tr.	0
			1750	72	80	190	479	857	12.4	8040	1.40	1.91	14.9	148
Recommended Dietary Allowances														
Woman (45 years)			2100	55	..	..	..	800	12	5000	1.0	1.4	10	70
Man (45 years)			2900	65	..	..	..	800	12	5000	1.4	1.6	14	75

[1] Average values for each food group have been computed according to the percentage distribution of food supplies as described in *Planning Food for Institutions*, Agriculture Handbook No. 16, U. S. Department of Agriculture, 1951.

Food values used are those published in *Composition of Foods—Raw, Processed, Prepared*, by B. K. Watt and A. L. Merrill, Agriculture Handbook No. 8, Bureau of Human Nutrition and Home Economics, Washington, 1950.

Calories and vitamin A have been rounded off to the nearest 5 and protein, fat, and carbohydrate to the nearest whole gram.

[2] Values for sodium are those naturally occurring in food before any additions have been made through processing and cookery. The values used are those from table 2 in *Sodium-Restricted Diets*, Publication No. 325, by a committee of the Food and Nutrition Board, National Research Council, Washington, D. C., 1954.

[3] The vitamin A value is reduced to 0 if an average of 1 ounce of liver per week is omitted.

[4] Includes farina, rolled oats, rolled wheat cereal, wheat meal, shredded wheat, puffed wheat, puffed rice. Some quick-cooking cereals to which disodium phosphate has been added and other dry cereals with added salt are omitted.

[5] Includes asparagus, broccoli, Brussels sprouts, cabbage, escarole, lettuce, lima beans (not frozen), okra, peas (not frozen), peppers, pumpkin, snap beans, winter squash, turnip greens. Excludes beet greens, carrots, chard, kale, spinach.

[6] Includes cauliflower, corn, cucumber, eggplant, onion, parsnip, radishes, summer squash, rutabagas, tomatoes. Excludes beets, celery, white turnips.

[7] Includes all fruits other than citrus—fresh, canned, or frozen.

Baking powder, baking soda, sodium acetate, sodium benzoate, sodium propionate, sodium alginate, sodium citrate, monosodium glutamate, and many other sodium compounds are used in food manufacture and home preparation so that it is essential that label information be noted before a processed food is used for a diet. The Food and Drug Administration now requires that foods intended for use in sodium-restricted diets must be labeled to indicate the number of milligrams of sodium in 100 g of the food and in an average serving of the food.[2]

Dietary restriction of sodium may be of no avail if sodium-containing barbiturates, sulfonamides, antibiotics, and salicylates are used. Patients need especially to be warned about the use of sodium-containing antacids or of sodium bicarbonate or salt used as a gargle or a dentrifice. A 2 g dose of sodium bicarbonate will contribute 547 mg of sodium.

Nutritive Adequacy of Sodium-Restricted Diets

Many sodium-restricted diets have, in the past, provided inadequate levels of calcium, riboflavin, and protein because of the omission of milk. Likewise, the exclusion of meat and eggs has tended to jeopardize satisfactory protein, iron, and vitamin B complex nutrition. The injudicious inclusion of certain vegetables and some sodium-containing processed foods in diets at the expense of essential protein, mineral, and vitamin-rich foods is scarcely warranted.

Sodium levels of 500 mg or more per day may permit completely adequate allowances of all nutrients for the man or woman, as will be noted in the calculations for a basic plan in Table I. When sodium levels of 250 mg or less are indicated, the use of sodium-deficient milk in place of regular milk still insures a nutritionally adequate diet. Sodium-deficient milk powder has been available for a number of years, but more recently a liquid sodium-deficient milk has become available from dairies in metropolitan areas; the latter is a product of excellent flavor practically indistinguishable from that of fresh milk.

The caloric level of the basic list of foods is sufficient for many afebrile sedentary patients, but the caloric intake may be increased or decreased by using more or less of low-sodium cereals and breads, fats, and potatoes. Fruits, sugars, and jellies may be used as desired when there is no caloric restriction.

A Meal Plan for the Daily Diet

The basic list of foods should be adjusted to the meal patterns of the individual patient. One such meal pattern is given here.

Suggested Meal Pattern	*Sample Menu*
All foods prepared and served without salt	
Breakfast	
Fruit, citrus—1 serving	Orange juice
Whole-grain or enriched cereal—1 serving	Shredded wheat
Milk for cereal and to drink—8 ounces	Milk—1 cup
Egg—1	Soft cooked egg—1
Unsalted bread—1 slice	Unsalted toast
Unsalted butter—2 teaspoons	Unsalted butter
Coffee or tea	Coffee Sugar, if desired Jelly, if desired
Luncheon or Supper	
Lean meat, poultry, or fish —2 ounces	Ground beef, rice, and tomato casserole
Potato or substitute	
Vegetable, green or yellow, raw or cooked—1 serving	Mixed green salad—lettuce, endive, cauliflower, green pepper, radish Unsalted French dressing
Unsalted bread—1 slice	Orange biscuits*
Unsalted butter—2 teaspoons	Unsalted butter; jelly
Fruit—1 serving	Strawberries
Milk—8 ounces	Milk—1 cup
Dinner	
Lean meat, fish, or poultry—2 ounces	Roast turkey — small serving Cranberry jelly
Potato or substitute	Mashed potato
Vegetable—1 serving	Buttered asparagus
Unsalted bread—1 slice	Unsalted roll
Unsalted butter—2 teaspoons	Unsalted butter

* Biscuits are leavened with sodium-free baking powder; orange juice is used as the liquid.

Fruit or dessert	Stewed apricots
Coffee or tea	Tea with sugar, lemon

Foods from Which to Choose	*Foods to Avoid*
Beverages	
Carbonated	Dutch process cocoa
Cocoa	Milk drinks except as specified on diet
Coffee	
Fruit juices	
Tea	
Breads and crackers	
Passover matzoth	Commercial bread, biscuit, pancake, or waffle mixes
Quick breads made with low-sodium baking powder or potassium bicarbonate	Salted breads
	Self-rising flours
Unsalted bread	Pretzels
Yeast rolls, waffles, griddle cakes	White and graham crackers
Cereals	
Cooked, unsalted farina, wheat meal, rolled oats, rolled wheat	All dry cereals except those listed as allowed
Puffed rice	Quick-cooking cereals which contain disodium phosphate
Puffed wheat	
Shredded wheat; muffets	
Macaroni, rice, spaghetti	
Cheese	
Washed cottage	All except as noted
Sodium-deficient Cheddar (dietetic)	
Desserts	
Custard, ice cream (homemade), and puddings using part of milk and egg allowance	Cakes, and cookies with regular baking powder or baking soda
	Commercial cake, cookie, sweetened gelatin, ice cream, and pudding mixes
Fruit tapioca pudding	Commercial ice cream
Fruit pies with unsalted crust	
Gelatin desserts with plain gelatin and fruit juice	
Cakes and cookies using sodium-free baking powder; salt omitted	
Egg	
Only 1 permitted	
Fats	
Unsalted butter or margarine	Bacon fat
	Commercial salad dressings
Vegetable shortening and oil	Cream except as part of milk allowance
Lard	
Unsalted salad dressings	Salted butter or margarine

Foods from Which to Choose	*Foods to Avoid*
Fruits	
Use without restriction except as noted	Those containing sodium benzoate as preservative
	Note: Apples, melons, and berries are avoided if there is a tendency to distention
Meat, poultry, fish	
Fresh or frozen beef, lamb, pork, veal, or rabbit	Brains or kidneys
	Canned, salted, or smoked meats or fish: bacon, chipped beef, frankfurters, ham, herring, luncheon meats, salt pork, sausage
Fresh or frozen chicken, duck, quail, turkey—especially white meat	
Liver—small serving once every 2 weeks	
Fresh cod, bass, halibut, flounder, salmon	Shellfish: clams, crabs, lobster, oysters, shrimp
Canned unsalted (dietetic) salmon or tuna	Frozen fillets or steaks of fish
Canned dietetic pack meats	
Milk	
Only in specified amounts	
Nuts	
Unsalted	Salted; peanut butter
Soups	
Unsalted broth or using allowed milk and vegetables	All canned
	Salted broth and cream soups
Sweets	
Jams and jellies	Jellies containing sodium benzoate
Sugar and candies (note label information—some contain salt)	Candies to which salt or other sodium compounds have been added
Vegetables	
Fresh, frozen, or canned without salt:	Canned unless canned without salt:
Asparagus	Beets
Broccoli	Beet greens
Brussels sprouts	Carrots
Cabbage	Celery
Cauliflower	Dandelion
Cucumber	Kale
Corn	Mustard greens
Eggplant	Spinach
Endive	Swiss chard
Lettuce	Sauerkraut
Lima beans (not frozen)	White turnips
	Potato chips

Foods from Which to Choose	*Foods to Avoid*
Okra Onions Parsnips Peas (not frozen) Peppers Sweet potato White potato Pumpkin Radishes Rutabagas String beans Squash of all kinds Tomatoes Turnip tops	*Note:* When there is distention, avoid dried beans, legumes, broccoli, Brussels sprouts, cabbage, cauliflower, cucumber, onion, green pepper, radish, rutabaga, turnips

Seasonings

Extracts: almond, lemon, maple, orange, peppermint, vanilla, walnut

Herbs: basil, bay leaf, chives, dill, marjoram, mint, oregano, parsley, rosemary, sage, savory, thyme

Spices: allspice, cinnamon, cloves, curry, ginger, nutmeg, paprika, pepper, dry mustard

Meat flavorings such as Kitchen Bouquet, A-1 sauce, Worcestershire sauce, soy sauce, tabasco sauce

Prepared mustard and horseradish

Vegetable salts: celery, onion, garlic, bouillon cubes

Gravy

Catsup, chili sauce, olives, pickles, relishes

Preparation of Food

The success of the sodium-restricted diet is determined by the patient's acceptance of the planned meals. Most spices, herbs, and other flavorings are low in sodium and may be used to provide variety in flavor. Many recipes can be used when the salt is omitted and when sodium-free leavening agents are used. It is necessary to use $1^1/_2$ teaspoons of sodium-free baking powder for each teaspoon of regular baking powder specified in a recipe. Sodium-free baking powder may be purchased or may be mixed in any drugstore according to the following formula:[3]

Potassium bicarbonate	39.8 g
Cornstarch	28 g
Tartaric acid	7.5 g
Potassium bitartrate	56.1 g

Potassium bicarbonate may be substituted for equal amounts of baking soda in any recipe.

Yeast-leavened breads, rolls, doughnuts, and waffles provide interesting variety.

Milk and eggs used in a recipe must be subtracted from the dietary allowance. It is also possible to substitute water for milk in most recipes by using 1 cup of water less 1 tablespoon for each cup of milk. Unsalted butter or vegetable fats are used in place of salted fats.

Raw fruits and vegetables should be given a prominent place in the diet since they contribute so much in flavor without the necessity for salt. Cooked fruits may be used in unsalted pastries.

A number of salt substitutes are available and have been accepted by many patients. They should not be used indiscriminately, and may be contraindicated altogether for patients with renal disturbances.

Variations of the Sodium-Restricted Diet

By referring to the basic diet calculations in Table I it will be observed that the following modifications can be made without difficulty:

250 mg sodium: substitute 2 cups of sodium-deficient milk for 2 cups of regular milk

250 mg sodium, 100 g protein: omit regular milk and egg; substitute 4 cups of sodium-deficient milk; add 4 ounces of meat

800 mg sodium, with added protein and calcium, as for children and in pregnancy: add 2 cups of regular milk

1200–1300 mg sodium: substitute 3 level teaspoons of salted butter (145 mg sodium) and 4 thin slices of salted bread (about 600 mg sodium) for unsalted bread and butter in basic pattern. Commercial bread and butter vary considerably in sodium content.

REFERENCES

1. Committee of the Food and Nutrition Board: *Sodium-Restricted Diets,* Publication 325, National Academy of Sciences—National Research Council, Washington, D. C., 1954.
2. *Federal Register,* July 1, 1954; 19 F. R. 3999.
3. *Planning Low Sodium Meals,* Newton Health Department, Newton, Mass., 1951.

Nutrition News

☆ News of activities in the field of clinical nutrition should be submitted to the Editorial Office of the JOURNAL.

Editorial Board Changes

We are pleased to announce that **Charles S. Davidson, M.D.,** has joined the Editorial Board of this JOURNAL. Dr. Davidson is an Associate Professor of Medicine at Harvard Medical School and Associate Director of the Thorndyke Memorial Laboratory, Boston. He is also a member of the Food and Nutrition Board of the National Research Council. He brings to the Editorial Board a wide experience in clinical nutrition.

We regret that our advisor in internal medicine, **Charles L. Brown, M.D.,** Dean of Hahnemann Medical College, will be unable to continue his connection with this JOURNAL, due to the pressure of duties connected with his new position as Dean of the Seton Hall University College of Medicine, now being organized.

Our new advisor in internal medicine is **William B. Bean, M.D.,** professor of Medicine, Iowa State University College of Medicine, Iowa City. Dr. Bean is well known for his wide interests and important clinical observations.

We are honored to announce the appointment as our contributing editor from England, replacing the late Sir Edward Mellanby, of **Robert A. McCance, M.D., F.R.C.P., C.B.E., F.R.S.,** Professor of Experimental Medicine, Medical Research Council and University of Cambridge. Professor McCance is one of Britain's most prominent nutritional investigators.

A new contributing editor is **Salvador Zubirán, M.D.,** Professor of Internal Medicine, Facultad de Medicina de México and Graduate School of Medicine, Universidad Nacional de México, and Director of the Hospital de Enfermedades de la Nutrición, México, D. F.

Vitamin E Congress

The Third International Vitamin E Congress will be held this year in Venice, from September 5 to 8, at the Cine Foundation, Island of San Giorgio Maggiore.

Papers will be presented on both experimental and clinical aspects of several general topics, one topic each day. Among the general and specific items to be discussed are the following:

VITAMIN E AND METABOLIC PROCESSES—Prof. Dr. U. Butturini, Istituto di Patologia Medica, Università di Bologna.

VITAMIN E AND ITS RELATIONS TO OTHER VITAMINS, HORMONES, AND ENZYME SYSTEMS—F. Verzár, Direktor des Physiologischen Instituts der Universitäts Basel.

Correlations between Vitamin E and other Vitamins—Dr. R. Beckmann, Kinderklinik der Westfälischen Wilhelms-Universität Münster.

Vitamin E in Obstetrics and Gynecology—Prof. G. Vecchietti, Direttore della Scuola Ostetrica, Vercelli.

Thyroid and Vitamin E—Prof. A. Costa, Ospedale Mauriziano, Torino.

Vitamin E and Blood Coagulation—Prof. P. Prosperi, Clinica Pediatrica, Università di Firenze, and Prof. P. De Nicola, Clinica Medica, Università di Pavia.

VITAMIN E AND CARDIOVASCULAR SYSTEM—Prof. A. Vannotti, Directeur de la Clinique Médicale de l'Université de Lausanne.

Arthritis and Vitamin E—Prof. A. M. Boyd, Director of the Surgical Clinic, University of Manchester.

Vitamin E and Vasculopathies of the Cutaneous System—Prof. M. Comel, Direttore della Clinica Dermosifilopatica, Università di Pisa.

Vitamin E and some Vasculopathies of the

Ocular Apparatus—Prof. E. Raverdino, Primario Oculista, Ospedale Maggiore, Milano.

VITAMIN E, NERVOUS AND MUSCULAR SYSTEM—Prof. G. Meldolesi, Direttore della Clinica Medica, Università di Palermo, and Prof. Ch. Le Roy Steinberg, Rochester General Hospital, Rochester, N. Y.

Community Nutrition Institute

Sponsored by the College of Home Economics, Syracuse University, and the Bureau of Nutrition, New York State Department of Health, the Ninth Community Nutrition Institute was held from June 20 to July 2 at Syracuse University. This annual two-week postgraduate course is attended by nutritionists, physicians, nurses, public health workers, and others. The distinguished speakers represent nutrition, medicine, psychiatry, education, and allied fields.

Among the subjects and speakers of this year's Institute are:

A REVIEW OF SOME OF THE CURRENT CONCEPTS OF NUTRITION—

Current Nutrition Problems—Robert E. Shank, M.D., Head, Department of Preventive Medicine and Public Health, Washington University, St. Louis.

Nutrition Aspects of Blood Formation—Frank H. Bethell, M.D., University of Michigan.

Nutritional Aspects of Cardiac Failure and the Relation of Coronary Disease to Cholesterol Metabolism—Michael G. Wohl, M.D., Chief of Human Nutrition, Hahnemann Medical College and Hospital, Philadelphia.

Sodium Metabolism—George J. Gabuzda, M.D., Assistant Professor of Nutrition, Cleveland City Hospital.

PROBLEMS IN GROUP FEEDING—

The Individual in the Group—Elias Savitsky, M.D., The Home for Aged and Infirm Hebrews of New York.

Food Technology—Impact of Manufacturing Methods on Nutrition—Carl R. Fellers, Ph.D., Head, Department of Food Technology, University of Massachusetts, Amherst.

Methods of Evaluation—John H. Browe, M.D., Director, Bureau of Nutrition, New York State Department of Health.

Wayne University Nutrition Conference

The First Annual Nutrition Conference was held in the Medical Science Building of Wayne University College of Medicine in Detroit on Tuesday, May 10, 1955. The meeting is under the joint sponsorship of Wayne University College of Medicine and the Michigan Nutrition Council. This year, the speakers were Dr. James M. Orten, Associate Professor of Physiological Chemistry at Wayne University College of Medicine, and Dr. J. B. Brown, Professor of Physiological Chemistry at the Medical College and Director of the Institute of Nutrition and Food Technology of Ohio State University. Dr. Orten discussed "Some Non-Dietary Factors affecting Nutritional Status"; Dr. Brown's subject was "The Relation of Nutrition to Public Health."

Coming — Special Issue

NUTRITION IN SURGERY—A SYMPOSIUM

I. S. Ravdin, M.D., Guest Editor

The issue of the Journal for November-December 1955 will contain an unusually interesting and practical symposium on nutrition in modern surgery. The guest editor is I. S. Ravdin, M.D., John Rhea Barton Professor of Surgery, University of Pennsylvania.

Nutritional Quotes

Trends in Nutrition

"Two areas of nutrition research seem to me to stand out for special emphasis. First, is the growing importance of mineral elements in regard to basic physiology at all levels, from plants to the human problems of health.

"Second, is the growing evidence that we must increasingly regard nutrients in terms of the *total* organism and the interplay or balance of all of the nutrients in the preservation of health on a life-span basis.

"These two trends may not seem new or revolutionary, but I think they merit emphasis, both with respect to current research and in their implications for education and practice. Vitamins, for example, are no less important than they were one or two decades ago when their discovery was so exciting, but you will note that most of the current work relating to vitamins deals with their interplay with other nutrients and the long-term relationships of these balances to protecting health. Single or acute deficiencies become less and less important."

—Charles Glen King. *Journal of the American Dietetic Association* 31: 225, 1955.

Shelf Life and Multivitamin Potency

"The potency of certain vitamins in some pharmaceuticals is markedly affected by shelf age. There are definite losses in potency in several products as early as eight months after manufacture. . . . Vitamins A and B_{12} seem to be particularly unstable in the liquids. . . . With the exception of pantothenic acid, vitamins in capsules are relatively stable in all factors. . . .

"Certain conclusions may be drawn from these data. The facts that many multivitamin products have begun to lose potency less than one year after manufacture, and that over 30% of products purchased at random on the retail market are past this age indicate that a large proportion of market samples must be below labeled claim in one or more vitamins. In fact, over 50% of the samples studied were deficient in one or more vitamins. What is equally important, the data indicate that existing control facilities are not as effective as they should be in the pharmaceutical industry. . . . It is suggested that an expiration date, conspicuously displayed on the label, would do much to ensure that the consumer obtains a product which meets label claims."

—J. A. Campbell and H. A. McLeod. *Journal of the American Pharmaceutical Association* 44: 267–9, 1955.

Physician, Starve Thyself

"Dietary restrictions for an acute emergency lasting only a few days, such as diabetic acidosis or a gastrointestinal upset, are easily tolerated, but if dietary restriction becomes a part of the treatment of a chronic disease numerous difficulties must be met. Because many physicians who prescribe such a diet do not appreciate this fact Rittelmeyer urges the physician never to prescribe a restrictive diet unless he has first tried it himself, and he quotes Dr. Irvine Page as saying that after placing himself on a low fat diet his blood lipids dropped (an expected but not necessarily beneficial change) but that also there was an impairment of his disposition and a marked constriction of his circle of friends. Fortunately these changes were reversible."

—Editorial. *Journal of the American Medical Association* 158: 123, 1955.

"Protein Diabetes"

". . . Experimental nephrosis can be looked upon as a 'protein diabetes.' The pattern of events in the 'protein diabetes' of experimental nephrosis may be pictured as follows: the uncontrolled loss of albumin in the urine leads the body to direct its metabolic efforts to a greatly increased fabrication of protein in the liver and perhaps other tissues. This is an adjustment of desperation, and since the protein cannot be retained, it is an 'unwise' process, much like the production of glucose in diabetes mellitus. The metabolic channeling of amino acids into the acceleration of protein synthesis depletes the store of carbohydrate. Under these circumstances, as in diabetes mellitus, the demands for cellular energy must be covered by fat."

—David L. Drabkin and Julian B. Marsh. *The Journal of Biological Chemistry* 212: 629–30, 1955.

Food Consumption and Caries Production

"Since Miller's time many workers have added their contributions to expand and develop his theory. The foodstuffs which have been singled out for the most extensive studies are refined white flour and sugar, more particularly the latter. The justification for intensifying investigations along these lines has come from the following four sources: the results of numerous field studies, which have pointed strongly to the importance of sugar consumption; the effects of diets low in carbohydrate on the caries rate in diabetic and other children under strict dietary control; studies of the structure, pathology and biochemistry of the plaque which forms on teeth; and advances in enzymology, which have shown how acid can be produced by bacterial action from carbohydrates."

—F. W. Clements. *The Medical Journal of Australia* 1: 298, 1955.

Smoking and Weight

"It would be idle to discuss weight reduction by diet without a word about the effect of smoking on weight. The chief disadvantage of smoking in excess, apart from its possible association with cancer, is that it irritates the throat and upper respiratory passages and predisposes to cough, catarrh, and bronchitis. Apart from its soothing effect and social uses, the main advantage of smoking is that it permits some people to maintain a reasonable figure without suffering the pangs of self-denial at meal times. Its effect in helping to keep down excessive weight is probably due to the fact those who smoke, eat and absorb less. Those who give up smoking are sometimes unjustly rewarded by a considerable increase in weight, and if they happen to be suffering from hypertension or cardiovascular disease, or any of the other conditions in which weight reduction is important, the effect may be serious. For this reason smoking and diet should always be considered together, and if for any reason, such as chronic cough, it becomes necessary to curtail a patient's smoking, he must be simultaneously warned to be careful that he does not eat more carbohydrate and fat, and to watch his weight carefully. Smoking in moderation may be of considerable value in helping to achieve or maintain a reduced weight, and in the potentially obese smoking habits should only be changed after careful attention to this aspect. The effects of over-nutrition may well outweigh the advantages of not smoking."

—Leslie Cole. *The Medical Press* 232: 529, 1954.

Food Timing after Trauma

"To ward off the criticism of the nutritional enthusiasts we must make clear our realization that good feeding of good foods to good-functioning gastrointestinal tracts is the key to recovery. Such feeding should be begun as soon after trauma as acceptance and absorption are evident; the gradual anabolic weight gain will then begin as soon as it can. But before the patient is ready to absorb, assimilate and synthesize, we do no good by forcing water and nutriments into him. We only produce a weight gain of water-logging, or the devastating complications of distention, gastric dilatation, vomiting and aspiration pneumonitis."

—Francis D. Moore. *Annals of Surgery* 141: 144, 1955.

Maternal Diet and Protein Malnutrition

"An immediate consequence of better feeding of the mother would be that she would produce ample milk daily and during a prolonged lactation. This increased daily yield would eliminate protein malnutrition in breast-fed infants, and the lengthening of adequate lactation would reduce mortality and morbidity among children in the second year of life, particularly those who are being weaned on to diets low in protein and relatively high in carbohydrate. Such an improvement in lactation would therefore contribute to eradication of protein malnutrition in children—a widespread and serious problem to which much attention has latterly been given."

—B. S. Platt. *The Lancet,* Nov. 6, 1954, p. 932.

Vitamins and Antibiotics Compared

"From a historical point of view, antibiotics were discovered and used as substances endowed with a *specific* action against certain microorganisms. They fulfilled a need that had existed from the beginning of this century for 'magic bullets' against germs. Antibiotics represent the crystallization of the etiologic thinking of Pasteur and Koch at the inception of microbiology. They grew from the philosophic notion that infections possess not only microbial origin but also etiologic specificity.

"On the other hand, the discovery of vitamins signified a great philosophic triumph of the period of positivism that began in the last quarter of the nineteenth century and which had as its greatest exponent Claude Bernard who, without knowledge of vitamins as such, recognized the importance of maintaining 'physiologic balance.' It then became of the greatest importance to understand nutrition biochemically and this materialized in the discovery of vitamins some forty years ago. . . .

"Vitamins entered medicine first as a therapeutic answer to the specific problems of nutritional deficiencies, but later they were incorporated into the art of good eating for the preservation of health. They therefore underwent an evolution not unlike that of the antibiotics, which were born as chemotherapeutic agents to treat acute or chronic illnesses and now are used as stimulants for animal development and growth and as food preservatives."

—Félix Martí-Ibáñez. *Antibiotic Medicine* 1: 249, 1955.

On Freedom to Write

"It is likewise as unreasonable what some assert, *That Printers ought not to print any Thing but what they approve;* since if all of that Business should make such a Resolution, and abide by it, an End would thereby be put to Free Writing, and the World would afterwards have nothing to read but what happen'd to be the Opinions of Printers."

—Benjamin Franklin

Reviews of Recent Books

The Vitamins: Chemistry, Physiology & Pathology, Vol. I, edited by W. H. Sebrell, Jr., and Robert S. Harris, Academic Press Inc., New York, 1954, pp. 676, $16.50.

The amazing growth of basic scientific knowledge in the broad field of nutrition, and its practical application over the past quarter of a century, are based on a voluminous and widely scattered literature which is difficult for anyone but an expert to evaluate. Knowledge has crystallized enough to warrant a critical and detailed appraisal of this important field. This has been effectively accomplished by the editors with the help of some twenty contributors, each an authority in some aspect of the subject.

The first volume of this work brings together an enormous body of information. It serves as a guide to areas of knowledge which are still new and complicated for many.

The reviewer was impressed with the easy readability and the good organization of this book. Each section is discussed by an expert in the field of chemistry, industrial production, biochemistry, pharmacology, estimation, occurrence, deficiency effects, and general requirements. Although the individual sections are particularly strong on chemistry and physiology, there is also an excellent discussion of the practical clinical aspects of each deficiency state. This volume deals with vitamin A and carotene, ascorbic acid, vitamin B_{12}, and biotin.

The value of the book is enhanced by its broad reference to all branches of the sciences represented, and it is not limited to the clinical aspects. It correlates available information as to the part vitamins play in the metabolism of the human organism.

This volume can be highly recommended and is certain to be a much consulted reference source. It contains a tremendous amount of scientific material which is concisely presented, clearly and accurately indexed, and extensively documented.

HAROLD JEGHERS

Sodium-Restricted Diets. The Rationale, Complications, and Practical Aspects of their Use, by a Committee of the Food and Nutrition Board, National Research Council, Publication 325, July 1954, pp. 71, $1.00.

Because of the importance of sodium restriction in medicine, the Food and Nutrition Board of the National Research Council appointed a subcommittee to review and summarize the clinical and dietary conditions for sodium-restricted diets.

This short but excellent report should be read by all whose interest brings them into contact with sodium metabolism. Normal physiology, dietotherapy, indications and contraindications are well covered. There are also useful chapters on planning sodium-restricted diets and on salt substitutes. The entire report is clear, succinct, authoritative, and practical.

Much of this material appeared in the *J. A. M. A.* in November 1954. Now, conveniently published as a separate monograph, it will be read profitably by clinicians, nutritionists, and dietitians. S.O.W.

Diet Manual of the Jewish Hospital of Brooklyn, edited by Charles Solomon, M.D., Nanette D. Robertson, M.A., and Mariam C. Tekulsky, B.S., Brooklyn, 1954, pp. 87.

This manual represents a distillation of dietary information and menus used in hospitals in general, and in the Jewish Hospital of Brooklyn in particular. In view of the limited space at their disposal, the authors have succeeded admirably.

The manual contains "routine" hospital diets, postoperative diets; high vitamin diet; low and high calorie diets; diabetic diets; sodium-restricted diets; low fat and low cholesterol diet; high and low protein diets; low purine diet, acidogenic diet, alkalinogenic diet; low potassium diet; high iron diet; diets in allergy; and diets used in pediatrics and obstetrics.

It is a pity that this manual could not have presented a few kosher diets designed for the orthodox Jewish patient. These would have added considerably to the usefulness of the volume. After all, the impact of eating "trefo" (non-kosher food) on the psyche of the orthodox Jewish patient must be considerable, and this may detract from the rapid recovery from illness. M.G.W.

The Biochemistry of the Nucleic Acids (second edition), by J. N. Davidson, John Wiley & Sons, Inc., New York, 1954, pp. 200, plates 4, $2.25.

This little book is the busy scientist's guide to the important but formidable field of the nucleic acids. About half of it describes the products of the degradation of nucleic acids, and the structure and

properties of polynucleotides. The remainder deals with nucleic acids in the cell, their metabolism and biological activity. The skill with which the important and interesting aspects were presented made the first edition a great success. The inclusion of much new work, some of it as recent as 1953, makes the new edition even more useful. J.G.R.

Practical Chromatography, by R. C. Brimley and F. C. Barrett, Reinhold Publishing Co., New York, 1953, pp. 128, $5.00.

Apparatus, methods, and applications of chromatography on paper and on columns by means of adsorption, partition, and ion exchange are described in this handbook by two chemists of the Low Temperature Station at Cambridge. The authors draw upon a considerable experience to describe their techniques, including many details and a number of tricks that may make the difference between sharp and unsharp separations. The approach is mainly practical, and the reviewer, whose experience with chromatography is not extensive, has obtained valuable help from it in study of a variety of biological materials. An appendix describes solvent mixtures, sprays, and R_f values. JOHN G. REINHOLD

Kwashiorkor, by H. C. Trowell, J. N. P. Davies, and R. F. A. Dean, Edward Arnold (Publishers) Ltd., London, 1954, pp. 308, $10.00.

In the underdeveloped countries of the tropical belt protein malnutrition is very widespread in children after the weaning period. In its tropical form, it has only recently been recognized by organized scientific medicine. Its designation as kwashiorkor, originally introduced in the medical literature by Cicely Williams, is very old, having been in common use among one African tribe for as long as tradition and memory serve. The authors of the present monograph are eminently qualified to treat the subject from personal experience and from all possible angles, Trowell being the clinician with long experience in the field, Davies a prominent pathologist, and Dean the nutritionist with excellent pediatric experience and biochemical training. The book is divided in several sections. The chapter of Trowell on the history of kwashiorkor represents an excellent and detailed review of all relevant publications for all corners of the earth. In addition to the clinical picture, pathology, biochemistry, prevention and treatment of kwashiorkor in infants and children, a special chapter describes what is believed to be protein malnutrition in adults. The clinical pictures in adults and children are similar and so are the pathological changes, but the authors concede that further work might disclose clear differences. The book closes with a chapter devoted to some of the possible implications of the problem of protein malnutrition in children and adults. With good logic and in the light of impressive circumstantial evidence, the authors discuss the possibility that "acute Kwashiorkor in childhood is but the advanced stage of a common variety of protein malnutrition from which many children suffer mildly during their early period of growth. Many adults in the tropics live, like the children, on diets which provide very little protein. It may be that the investigation of the effects of protein malnutrition will throw light on many facts which at present lack explanation, such as the curious frequency of certain diseases among African adults and the variety of others."

This and similar interpretations of factual information add greatly to the value of the monograph, not only for research workers actively engaged in the specific problem of kwashiorkor, but also for those with wider horizons, in particular for clinicians with interest in geographical pathology, for all nutritional research workers, for biochemists, and, to some extent, for everybody who appreciates the world-wide implications of malnutrition, population pressure, cultural anthropology, and world economy.

A large number of well reproduced illustrations adds to the value of this excellent book.

PAUL GYÖRGY

Tea—A Symposium on the Pharmacology and the Physiologic and Psychologic Effects of Tea, edited by H. J. Klaunberg, The Biological Sciences Foundation, Ltd., Washington 7, D. C., 1955, pp. 64, $1.00.

A symposium on the pharmacologic, physiologic, psychologic, and clinical aspects of tea was recently held at the New York Academy of Sciences. This little booklet contains six papers presented at the meeting and a reprinting of two significant articles on the same subject.

As pointed out by the various authoritative contributors, it is striking that there has been little organization of research and literature on this ancient and widely used household beverage. With essentially no medical contraindications, and by virtue of palatability, ease of preparation, and low cost, tea certainly merits even more recognition than it now enjoys.

This booklet summarizes concisely and attractively the essential facts about the pharmacology (of caffeine and tannins), nutritional aspects, medical uses, and psychological effects of tea. It will be of unusual interest to all who are professionally concerned with human nutrition and physical fitness. A.E.S.

Methods for Evaluation of Nutritional Adequacy and Status, published by the Advisory Board on Quartermaster Research and Development, 1954, Washington, D. C., pp. 313. Copies available from Quartermaster Food and Container Institute for the Armed Forces, Chicago 9, Ill.

In 1954 a symposium was arranged under the joint sponsorship of the Medical Nutrition Laboratory, Office of the Surgeon General, Department of the

Army, and the Nutrition Division of the Quartermaster Food and Container Institute for the Armed Forces. A group of national authorities discussed methods of evaluation of nutritional adequacy and nutritional status. This paper-covered book contains the proceedings—the papers and the following discussions—of this fine meeting.

As an indication of the caliber of the presentations, we may note papers by such investigators as King, Mitchell, Cannon, Allison, Bessey, Goldsmith, Darby, Snell, Wintrobe, Brožek, Elvehjem, Maynard, Sebrell, Jolliffe, and Grossman, and discussions by Kark, Youmans, and Horwitt, to mention only a few. The subjects range from rat repletion methods for the evaluation of protein adequacy to blood levels as indicators of vitamin adequacy. Nutritional status of populations by survey methods, physical performance, and body composition are among the several topics on the agenda.

Because of the significance of the subject, the importance of the observations discussed, and the stature of the participants, this book is of considerable value to all interested in the field of nutrition. S. O. W.

From the Workshop of Discoveries, by Otto Loewi, University of Kansas Press, Lawrence, 1953, pp. 58, $2.00.

In three short but charming essays, Otto Loewi shares much of his mature reflection on the study of medicine, the ways in which medical discoveries are made, and problems of adrenal function. All physicians will enjoy these simply written but deeply penetrating analyses of important facets of medicine. There is a great need for more "lectures" of this type, but we are indeed grateful for these thoughts of a wise and humane scientist. S.O.W.

Books received for review by the *American Journal of Clinical Nutrition* are acknowledged in this column. As far as practicable, those of special interest are selected, as space permits, for a more extensive review.

The Low Sodium Cook Book (Sunkist Growers spec. ed.), by A. P. Smith and D. Callahan, Little, Brown and Company, Boston, 1953, pp. 477.

Ion Exchange and Adsorption Agents in Medicine, by G. J. Martin, Little, Brown and Company, Boston, 1955, pp. 333, $7.50.

Les Gastrites, by F. Moutier and A. Cornet, Masson et C^ie^, Paris, 1955, pp. 404, bound 4.400 fr., paper 3.800 fr.

Antibiotics Annual 1954–1955, edited by H. Welch and F. Martí-Ibañez, Medical Encyclopedia, Inc., New York, 1955, pp. 1154.

Medical Progress, edited by M. Fishbein, McGraw-Hill Book Company, Inc. (Blakiston Division), New York, 1955, pp. 346, $5.00.

Methods for Evaluation of Nutritional Adequacy and Status, by H. Spector and M. S. Peterson, Quartermaster Food and Container Institute for the Armed Forces, pp. 313, 1954.

The Care of Your Skin, by H. Lawrence, Little, Brown and Company, Boston, 1955, pp. 95, $2.50.

Abstracts of Current Literature

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VITAMIN A METABOLISM

The principal dietary sources of vitamin A, in addition to milk and eggs, are the yellow vegetables which provide the precursor of the vitamin, carotene. Supplementation of the diet, especially in infancy, is required with the provision of vitamin A in the form of fish oil concentrates. It is important that studies similar to that which follows be conducted in order to determine the availability and utilization of carotene derived from foods rather than from the purified products.

The Utilization of Carotene. II. From Sweet Potatoes by Young Human Adults. W. H. James and M. E. Hollinger. *J. Nutrition* 54: 65, 1954.

The importance of carotene as a source of vitamin A in the average diet in the United States is well recognized. Numerous absorption studies involving doses of purified carotene have been reported, but no report from an American laboratory has appeared in the literature on the utilization in the human of the carotene in any vegetable as estimated from fecal excretion.

The amounts of carotene excreted in the feces associated with three basal diets essentially devoid of carotene and vitamin A but otherwise adequate, and the fecal carotene following the use of these same diets supplemented with sweet potatoes, were measured for two months, using a group of 3 women and 5 men, 18 to 28 years of age. Protein of animal origin was furnished by lean meat and non-fat dry milk solids. The three diets supplied each subject daily with the following amounts of these two items: diet 1, 150 g meat (raw wt) and 12 g milk solids; diet 2, 150 g (raw wt) and 120 g milk solids; and diet 3, 300 g (raw wt) and no milk solids. The diets were fed 9 days each in sequence; then, for 4 days, diet 1 was again in effect.

Following the 31-day depletion period, diets 1, 2, and 3 were fed, supplemented daily with 60 g sweet potatoes which provided 3500 micrograms carotene/subject/day, again in sequence for three 9-day periods. The feces were collected quantitatively daily, composited into three-day specimens, and analyzed for carotene. For the group of 8 subjects, the average amounts of fecal carotene associated with supplemented diets 1, 2, and 3 were, respectively, 55.9, 57.6, and 48.9 of the carotene ingested in the sweet potatoes. The overall average value, which represented 216 subject-days, was 53.8 ± 1.6 per cent.—B. SURE

The principal storage depot for vitamin A is the liver, while the organs involved in its production from carotene are probably the intestine and liver. The dynamics of vitamin A transport and storage are of importance in nutritional studies, because various diseases have been associated with abnormalities of its serum and tissue concentration.

Studies on the Absorption, Deposition and Depletion of Vitamin A in the Rat. E. G. High. *Arch. Biochem. & Biophys.* 49: 19, 1954.

This report evaluates experiments designed to determine the path vitamin A follows after absorption with special regard to kidney and liver vitamin A deposition. Vitamin A-deficient albino rats were fed approximately 100 μg of vitamin A dissolved in cottonseed oil. The rats were sacrificed at 18 time intervals ranging from 2 hours to 30 days and the liver,

kidneys, and plasma analyzed for vitamin A.

A plasma value of approximately 18–30 μg per cent of vitamin A was obtained before liver storage occurred, indicating that physiological needs must be satisfied before liver deposition. Vitamin A first appeared in the blood, then the liver, and finally the kidneys. During depletion, hepatic vitamin A decreased and kidney vitamin A increased until the liver-to-kidney ration of vitamin A was approximately unity. It appears that after the plasma vitamin A reached a level of approximately 30 μg/per cent it was resistant to change, even under depletion conditions, and showed no significant decrease until the liver and kidneys were nearing exhaustion of the vitamin.—M. K. HORWITT

Intracellular Distribution of Vitamin A Esterase Activity in Rat Liver. J. Ganguly. *Arch. Biochem. & Biophys.* 52: 186, 1954.

Liver homogenates from vitamin A-depleted rats were fractionated by differential centrifugation at 5° C. Incubation experiments were carried out on the whole homogenate, nuclei, mitochondria, microsomes, and the supernatant fractions. The two forms of vitamin A (vitamin A alcohol and vitamin A acetate) were separated and determined quantitatively. Nearly all of the vitamin A esterase activity was found to be in the microsomal fraction, whose activity compared very well with that of the whole homogenate. The nuclear, mitochondrial, and supernatant fractions were inactive.—M. K. HORWITT

Absorption of the fat-soluble vitamin A is primarily into the lymphatics of the small intestine, and its release into the blood stream is apparently stimulated by feeding. This may have important implications in the evaluation of the vitamin A absorption test for pancreatic function.

The Effects of Eating and of Sham Feeding upon the Absorption of Vitamin A Palmitate in Man. A. I. Mendeloff. *J. Clin. Investigation* 33: 1015, 1954.

Five normal subjects failed to show a rise in serum vitamin A after oral administration of 105,000 micrograms of vitamin A palmitate in corn oil. However, the feeding of a meal two hours after the test dose activates mechanisms which help "absorption," so that the serum level promptly rises. Sham feeding is almost as effective as food ingestion, while atropine delays but does not prevent this phenomenon. Those subjects, 13 of 106, who failed to show a rise in serum vitamin A level when fed two hours after the test dose, all had steatorrhea.

From the reported observations, it appears that gastric emptying or secretion are not involved. Neither are other intraluminal factors concerned, as determined by the isolated jejunum. The mechanism is probably as follows: ingested fatty products, including vitamin A, remain for hours in the lacteals. The act of eating (as well as parenteral or oral hypertonic saline) produces a sudden contraction of the lacteals which floods the blood with vitamin A. —S. O. WAIFE

The generally held opinion that xerosis of the conjunctiva, known as Bitot's spots, is a manifestation of vitamin A deficiency alone has been contradicted by several investigators. This finding is now regarded as a relatively non-specific condition occurring in malnutrition, although vitamin A deficiency may be a predisposing factor.

Study of 22 Malnourished Patients with Bitot's Spots. K. W. Ascher. *Am. J. Ophthal.* 38: 367, 1954.

Among 642 patients with definite or suspected avitaminosis, 22 had Bitot's spots. This is a form of xerosis of the conjunctiva in which a fine adherent secretion is found in the dry conjunctiva at the limbus, usually on the temporal side of the cornea. Scrapings and cultures revealed corynebacteria xerosis. Most of the subjects apparently were suffering from a niacin deficiency, with riboflavin deficiency next in frequency. The accepted opinion that vitamin A deficiency plus general malnutrition is responsible for this sign could not be confirmed or denied. The relative frequency of vitamin deficiencies was greater in the small group with Bitot's spots than in others in the same clinic. It would appear that inadequate nutrition predisposes to the condition, rather than vitamin A deficiency alone.—S. O. WAIFE

In experimental animals, extensive damage to the nervous system occurs as a result of vitamin A deficiency. This condition has no counterpart in man. The increased spinal fluid pressure in the vitamin A-deficient animals prior to neurological lesions suggests a permeability change in the blood-brain barrier.

Effect of Vitamin A Deficiency on the Cerebrospinal Fluid Pressure of the Chick. D. H. M. Woollam and J. W. Millen. *Nature* 175: 41, 1955.

Chicks were reared on a diet containing no detectable provitamin A. Compared to healthy chicks fed on a full diet which have a cerebrospinal fluid pressure of less than 100 mm of water, they showed mean pressures of 108 at 30 days, rising to 212 at 125 days. There was no visible sign of neurological disease at the time the pressures were measured, nor of any bony abnormality.

The findings indicate that a raised cerebrospinal fluid pressure is one of the earliest manifestations of vitamin A deficiency in experimental animals and may appear before neurological disturbance becomes evident.—F. E. HYTTEN

Congenital Hydrocephalus Due to Experimental Hypovitaminosis A. J. W. Miller, D. H. M. Woollam, and G. E. Lamming. *Lancet* 2: 679, 1954.

Congenital hydrocephalus was produced in 47 out of 51 young born in 12 litters to 11 rabbit dams which were maintained for at least 14 weeks before mating and during pregnancy on a diet containing no detectable carotene.

In 4 of the litters, the 13 young were stillborn or died within 24 hours, and in 8 litters the remaining 38 young were recovered by necropsy on the 29th day of gestation.

The primary cause of the hydrocephalus was not determined, but the evidence suggested that the determining factors may be an overproduction of cerebrospinal fluid, combined with a relative insufficiency of the cerebral aqueduct.

The findings are presented in detail and there is an extensive discussion of the etiology of hydrocephalus which should be read in the original by workers in this field.—F. E. HYTTEN

Numerous cases of hypervitaminosis A have now appeared in the literature. In children, the disease is characterized by hyperirritability, swellings, and pain over the long bones, coarse sparse hair, dry skin, and bony exostoses. An acute form of the disease is described in the following abstract.

Acute Hypervitaminosis A. C. Hooft. *Maandschr. kindergeneesk.* 22: 271. 1954.

Protrusion of the fontanelle was observed in four infants (age 4 to $5^1/_2$ months) who had received large amounts of vitamin A (100,000–200,000 units), usually with simultaneous administration of vitamin D (100,000–600,000 units). The blood level of the vitamin was determined in three of the four infants. In only one was it elevated (at 24 hours post administration of the vitamin); in the other two, it was normal (at 48 hours post administration). Protrusion of the fontanelle appears to be the most constant of the signs of intracranial pressure which have been observed in hypervitaminosis A. The author ascribes the acute condition to a combination of factors: age of subject; gastrointestinal absorption; liver and reticulendothelial system reactivity; transient hypervitaminemia; and the nature of the preparation itself. —C.-J. HOWELL

In experimental animals, hypervitaminosis A in the female has led to the appearance of an increased rate of fetal death and congenital abnormalities.

Congenital Anomalies in the Rat Produced by Excessive Intake of Vitamin A During Pregnancy. S. Q. Cohlan. *Pediatrics* 13: 556, 1954.

This article briefly reminds one of the previous contributions which have demonstrated the influence of deficiency states, disease processes, hormones, and physicochemical agents on the developing fetus. The authors present significant data on the influence of an excess of vitamin A on fetal differentiation in rats.

Two hundred-ten mated female rats received from 15,000 to 75,000 IU of vitamin A, ranging from a single dose to daily doses from the second to the 16th day of gestation. One hundred controls were fed equal volumes of sorethithan monolaurate diluent. The pregnancies were terminated on the 20th to 21st day.

Marked litter failure resulted from a single dose of 75,000 IU of vitamin A on the eighth and especially on the tenth day, and from repeated dosages ranging from 25,000 to 35,000 IU for variable periods from the second through the sixteenth day. Fifteen thousand IU had no apparent adverse effect. When the litters are carried to term, a variety of congenital anomalies occurred which include encephaly, eye malformations, cleft palate, shortening of the mandible and maxilla, and spina bifida with meningocele and hydrocephalus.

Six rats were mated the second time and received no vitamin A during this gestation. All of the offspring were normal.

In discussing the possible mechanism of this effect, the authors mention that excess quantities of this vitamin may act as an antienzyme or antimetabolite during a critical period of development and produce congenital malformation.

No effort was made to transpose these results to humans; however, the authors justifiably caution against the indiscriminate use of excessive quantities of vitamins during pregnancy.—J. N. ETTELDORF

The effect of vitamin A upon chondroitin sulfate in developing bone has been shown to be that of increasing its rate of formation and turnover as measured by the sulfate label. A similar increase in sulfate utilization in skin may be related to mucopolysaccharide formation in this tissue.

Influence of Excess Vitamin A on the Sulphate Metabolism of Chick Ectoderm Grown in Vitro. H. B. Fell, E. Mellanby, and S. R. Pelc. *Brit. M. J.* 2: 611, 1954.

When the presumptive epidermis from a 6–7 day chick embryo is grown in a medium containing excess vitamin A, it differentiates not into the squamous keratinizing epithelium of the skin (as in a normal medium) but into a mucous secreting epithelium, often ciliated.

Two such explants, one in normal medium and one in a medium containing excess vitamin A, had radioactive sulfur in the form of $Na_2{}^{35}SO_4$ added to the culture. In the "normal" explants, sulfur was taken up similarly to squamous epithelium *in vivo*, i.e. little

in the epithelium and none in the keratin. In the explants grown in excess vitamin A, the utilization of sulfate is comparable to that of a normal mucous membrane in the body, sulfate being found in high concentration in the secretory cells.—F. E. HYTTEN

RIBOFLAVIN METABOLISM

The vitamin riboflavin is found within the cell as a mono- or dinucleotide in combination with specific proteins forming enzymes which function in cellular oxidation reactions. Milk represents the chief source of the vitamin; liver, yeast, and green leafy vegetables are also good sources. The clinical manifestations of riboflavin deficiency involve principally the ectodermal structures, with cheilosis, glossitis, mucosal fissuring, corneal vascularizations. The laboratory evaluation of this deficiency with loading tests and blood level determinations remains controversial, so that studies such as the following in normal subjects are of importance.

Riboflavin Metabolism of Women on Controlled Diets. Mei-Ling Wu, E. Warren, and C. A. Storvick. *J. Nutrition* 51: 231, 1953.

This is a report of a study to determine the variation in daily fasting serum riboflavin, as well as in urinary riboflavin and creatinine, of women on a controlled diet. Riboflavin metabolism was studied in 7 adult women ranging in age from 22 to 44 years on a controlled diet which met the recommended allowances of the National Research Council. Daily fasting values averaged 3.21 μg per cent for 207 determinations of serum total riboflavin and 1.41 μg per cent for 207 determinations of serum free riboflavin. The free riboflavin values include any flavin mononucleotide which was present in the serum. Urinary excretions of riboflavin varied from 315 to 467 μg per 24 hours, and ranged from 27 to 40 per cent of the riboflavin intake.

The effects of an oral test dose of riboflavin were studied. The concentration of riboflavin in the serum reached a peak in one-half to one hour after the test dose and approached the fasting level in 5 hours. The peak in urinary excretion of riboflavin occurred one hour after the test dose and by the fifth hour the excretion of riboflavin was about the same as the one-hour fasting excretion.—B. SURE

The vitamin affects the tissue enzyme concentrations of enzyme other than the dehydrogenases. Both xanthine oxidase and D-amino acid activity are increased by riboflavin administration.

The Relationship Between Dietary Riboflavin Concentration and the Tissue Concentration of Riboflavin-Containing Coenzymes and Enzymes. L. E. Decker and R. U. Byerrum. *J. Nutrition* 53: 303, 1954.

It has been well established that a metabolic function of riboflavin is its participation as the coenzymes flavin-adenine dinucleotide (FAD) or flavin mononucleotide (FMN) in several oxidative reactions. In this investigation it was found that the riboflavin intake necessary to maintain maximum tissue levels of riboflavin coenzymes varies with the organ and the particular coenzyme. In general, an intake of 30 micrograms per day was required to give maximum FAD and FMN concentrations in all tissues.

The FAD and FMN + free riboflavin concentrations in brain were almost independent of the amount of riboflavin in the diet. However, in kidney and liver, coenzyme concentrations were almost doubled when the intake was increased from no added riboflavin to 30 μg per day. In the heart muscle of riboflavin-deficient animals, the FAD concentration and the FMN concentration were about the same, whereas in kidney and liver tissues FMN concentration was less than the FAD content.

The liver enzymatic activity depended on the riboflavin intake in the same manner as the coenzyme concentration. Both D-amino acid oxidase and xanthine oxidase activity increase with increased intake of riboflavin. Xanthine oxidase activity reached a maximum with an intake of about 30 μg of riboflavin per rat per day. D-Amino acid oxidase activity, on the other hand, increased up to an intake of over 60 μg riboflavin per rat per day.—B. SURE

An interesting mechanism for the development of a conditioned type of riboflavin malnutrition is proposed in the following report.

Ariboflavinosis Due to Amebiasis. A. K. Mukherjee. *Indian M. J.* 49: 8, 1955.

A case of clinical "ariboflavinosis" is reported in a 38-year-old male with active amebiasis but without diarrhea. Large does of oral riboflavin and a vitamin B-complex syrup were ineffective in clearing up the glossitis, cheilosis, and stomatitis. Soon after the start of vigorous anti-ameba therapy, including emetin, vioform, and sulfaquanidine, and parenteral vitamin B complex, there was a rapid recovery in the clinical manifestations of the nutritional deficiency.

In vitro studies on the ameba cultured from this patient indicated a more rapid growth in the presence of vitamin B complex in the culture medium. The author suggests that the *E. histolytica* organisms were thriving on the vitamin B complex found in the intestine and until they were removed by specific therapy they took up their full quota, producing clinical riboflavin deficiency in the patient.

This hypothesis has not been proved, but it is intriguing and suggests that vitamin utilization by parasites might be a fruitful subject for research. There are large areas in the world where this may be highly significant.—S. O. WAIFE

In patients with impairment of the cellular phosphorylative processes, the administration of phosphorylated products such as cocarboxylase, adenosine triphosphate, and, as in the following report, phosphorylated riboflavin, has been recommended. The presence of phosphatases within the blood which are capable of rapidly dephosphorylating the phosphate esters gives rise to some doubt as to whether the intravenous administration of these substances could effectively by-pass the esterification mechanism. Further confirmation of this work should be sought before recommendations can be made for the use of these substances in treatment.

Contribution to the Study of the Variations in Carbohydrate Metabolism Induced by Riboflavin and by its Monophosporic Ester. L. L. Barbieri and M. A. Brunelli. *Intern. Ztschr. f. Vitaminforsch.* 25 (2): 157, 1954.

Before the vitamins supplied in food or therapeutic preparations can be converted into their active form, they must undergo enzymatic transformations. These conversions apparently take place within the cells, particularly those of the intestinal wall and the hepatic parenchyma. Because of their property of entering into the structure of coenzymes, all the vitamins of the B complex play an indispensable role in the endogenous metabolism of amino acids, lipids, and particularly carbohydrates.

The coenzymes for riboflavin are two: alloxazine-mononucleotide (AMN), a phosphoric ester, and alloxazine-adenine-dinucleotide, formed by the union of adenosine-5-phosphoric acid with the phosphoric ester by means of a pyrophosphate bond. The chief role of riboflavin is that of regulating oxidation-reduction processes, i.e., of donating and removing hydrogen. The enzymes of riboflavin (among others) are required for the step pyruvic acid $\rightleftharpoons$ lactic acid, at which point pyruvic acid is decarboxylated and CO_2 liberated.

These facts, together with recent data on the effect of riboflavin on blood lactic acid and pyruvic acid, inspired this investigation of the possible differences in the behavior of blood sugar, pyruvic acid, and lactic acid after the intravenous injection of (a) simple riboflavin and (b) phosphorylated riboflavin (AMN). Included in the experiment were both healthy subjects and patients with heart disease (most of them in decompensation) and concomitant hepatic insufficiency. Cardiac decompensation often leads to disturbed carbohydrate metabolism, and since the liver is an important site of phosphorylation, insufficiency of this organ might be expected to result in a relative inability to transform vitamins into the active form.

A total of 22 subjects were studied: 10 healthy persons and 12 patients with heart disease. After determination of fasting morning levels of blood sugar, pyruvic acid, and lactic acid, 5 of the healthy subjects were given 20 mg riboflavin intravenously; the other 5 received 20 mg of AMN. At hours 1, 2, and 3 after the injection, the blood levels of the same constituents were again determined.

The 12 patients with heart disease were handled in the same way: 4 with riboflavin; 4 with AMN, and the remaining 4 with both (separately). All were in the fasting state and at complete bed rest.

Healthy subjects responded similarly to both substances: blood sugar was barely affected; pyruvic acid increased after one hour, falling again in the following 2 hours; lactic acid decreased progressively.

In the patients with heart disease, the blood sugar response to riboflavin and AMN did not differ substantially from that of healthy persons, although the response was more irregular with riboflavin than with AMN. There was wide variation and considerable inconsistency in the response of both pyruvic and lactic acid to riboflavin administration. However, when the monophosphoric ester (AMN) was administered, responses corresponding to those of the healthy subjects were obtained in the patients with cardiac conditions.

These findings seem to demonstrate, if indirectly, that in patients with cardiac decompensation and concomitant hepatic insufficiency the capacity to phosphorylate riboflavin is indeed impaired. The normal organism is apparently capable of esterifying all or most of a 20-mg dose of riboflavin within 60 minutes. This rapid rate of phosphorylation is not achieved by patients with heart disease, and the action of riboflavin in the normal organism can be duplicated in such patients only by its monophosphoric ester. It is concluded, therefore, that phosphorylated riboflavin is to be preferred to simple riboflavin in conditions such as cardiac decompensation, in which there is interference with normal vitamin phosphorylation.—C.-J. Howell

Among the clinical manifestations of riboflavin deficiency, those affecting the eye may be of some importance, as indicated in the following study.

Contribution to the Ocular Manifestation of Riboflavin Deficiency. K. Irinoda and S. Sato. *Tohoku J. Exper. Med.* 61: 93, 1954.

From clinical and experimental observations on riboflavin deficiency, the authors conclude that in this deficiency there are degenerative vacuolar processes, compensatory vascularization, and reactive chronic inflammation in avascular or hypovascular areas such as the cornea, or inner layer of the retina. These ocular manifestations of ariboflavinosis occur with considerable frequency and are "pathognomonically reliable."—S. O. Waife

NOTES ON FOLIC ACID

Not only does folic acid fail to protect against the neurological complications of pernicious anemia, but it may hasten the development of combined degeneration of the spinal cord seen in this disease. Failure to establish the cause of anemia and the use of "shotgun" therapy for these conditions may lead to serious consequences if folic acid is inappropriately used in combination with other agents.

Dangers of Polypharmaceutical Preparations Containing Folic Acid. C. P. Lowther. *Brit. M. J.* 1: 564, 1954.

A case is described in which a proprietary preparation containing iron, vitamin B, extract of raw liver, and folic acid was given to a 46-year-old woman with an anemia which had not been accurately diagnosed. In fact, it was pernicious anemia. Some improvement of the blood condition occurred, but this was accompanied by the rapid development of combined degeneration of the cord. The patient improved rapidly on orthodox treatment.—F. E. HYTTEN

Folic acid (pteroylglutamic acid) is the precursor of the citrovorum factor (folinic acid). The latter substance is formed primarily in liver tissues as a metabolite of the vitamin, folic acid. Ascorbic acid has been shown to favor this conversion.

On the Metabolic Alteration of Pteroylglutamic Acid. C. A. Nichol. *Proc. Soc. Exper. Biol. & Med.* 83: 167, 1953.

Previous studies had suggested that anaerobic conditions might favor the formation of citrovorum factor from pteroylglutamic acid by liver slices. The present study demonstrated rapid formation of the citrovorum factor when homogenates of rat or chick liver were incubated with pteroylglutamic acid under an atmosphere of nitrogen; and acceleration of this by the presence of ascorbate in the incubation mixture. Little citrovorum factor was formed when the incubation was carried out under oxygen.—L. W. KINSELL

Folic acid is involved in the supply and utilization of single carbon groups such as formate or methyl moieties in the metabolism of glycine, choline, methionine, tyrosine, and other amino acids. In this Connection, it is involved in nucleoprotein metabolism. The influence of this vitamin upon enzyme activity in tissues involved in these processes continues to engage the attention of investigators.

A Study of the Oxidation of Formic Acid in the Folic Acid-Deficient Rat. B. Friedmann, H. I. Nakada, and S. Weinhouse. *J. Biol. Chem.* 210: 413, 1954.

Rats rendered deficient in folic acid by exclusion of the vitamin from the diet oxidized injected formate to about half the extent displayed by rats on normal or folic acid-supplemented diets. The differences observed in the oxidation of injected formate were very significant, since the highest rate of formate oxidation exhibited by a deficient rat was substantially below that of any of the normal animals, and the reversal effected by the folic acid supplementation was prompt. Differences in rates of absorption or excretion of the injected substrate were ruled out as possible explanations for the impairment of formate oxidation.

In vitro studies demonstrated a two-fold lowering of formate oxidation in heart, kidney, and liver homogenates prepared from folic acid-deficient rats. A previous study had shown that the magnitude of oxidation of formate by rat tissues *in vitro* paralleled their catalase activity. The authors had anticipated finding that catalase was concerned with the physiological oxidation of formate; however, the catalase activity, which is relatively low in normal kidney and heart, was not changed appreciably in the corresponding deficient tissues.

A subsequent paper by S. Weinhouse and B. Friedmann, *J. Biol. Chem.* 210: 423, 1954, discusses the formate production from C^{14}-labeled glycine, choline, methionine, and sarcosine in normal and folic acid-deficient rats.—M. K. HORWITT

Folic acid is a necessary factor in hematopoiesis, an effect which can be reversed by the use of the appropriate metabolic antagonists.

Effect of a Folic Acid Antagonist (9-Methyl PGA) Upon Rats Exposed to High Altitudes. P. D. Altland and B. Highman. *Am. J. Physiol.* 176: 1, 1954.

Rats were exposed to simulated altitudes of 18–25,000 feet for four hours daily. Those fed the folic acid antagonist 9-methyl-pteroylglutamic acid had the same survival time as normal controls. There were evidences of interference with hematopoiesis in marrow in 7 days and blood in 20–40 days. These were not changed by exposure to altitude. Altitude polycythemia was prevented if folic acid antagonist was started 22 days before exposure. If polycythemia developed first, it could be reduced but not eliminated.—M. J. OPPENHEIMER

ITEMS OF GENERAL INTEREST

Breast Feeding and the Prevention of Infant Malnutrition. B. S. Platt. *Proc. Brit. Nutr. Soc.* 13: 94, 1954.

Malnutrition contributes to the appallingly high death rates still to be found in many parts of the world among infants and young children and is the cause of much sickness and ill-health, some of which

persists into later life. The recognition of the ubiquity of malnutrition in infants and young children has led to the present interest in infant-feeding practices.

Provision for the health and well-being of the child should, of course, begin with antenatal care, and a long and ample lactation will partly depend on the condition of the mother during pregnancy. The place of the mother and the importance of her nutrition in relation to infant feeding is stressed.

Practices in the early period of infancy differ throughout the world. Infant feeding should begin when the mother is secreting colostrum. This period is of paramount importance in the establishment of lactation. Psychological, endocrine, and other factors are involved in the "let-down" of the mammary secretion, and a satisfactory mother-infant relationship is necessary to secure this. The competence with which the infant suckles is also important, since the volume of milk secreted is proportional to the degree of emptying of the breast. If for any reason the baby is not able to empty the breast properly, then simple manual expression is necessary. There seems to be no doubt that the correct procedure is to put the infant to the breast at intervals during the first few days, usually ten or twelve times daily. He thus obtains colostrum which has some nutritional value and possibly contains immune substances; the quantity of colostrum and early milk secreted is sufficient to provide him with the amount of food and fluid he requires at this stage—about $^{3}/_{4}$ oz milk/lb body-weight on the fourth day and $1^{3}/_{4}$ oz/lb body-weight on the seventh to the eighth day.

For some years a rigid feeding schedule has been in vogue in this country and there are still many advocates of this system. However, the so-called "self-demand system," which is elastic and leads ultimately to a regular rhythm characteristic for the infant, has now been introduced, largely as a result of observations in the United States of America.

The child should be accustomed to taking foods other than breast milk for two to three months before breast feeding ceases, and, in individual instances, some breast feeds might be continued for longer than nine months. In deciding on the duration of breast feeding, an all-important factor must be the diet available to the child after breast feeding has ceased.—B. Sure

Kwashiorkor Syndrome in a 3-months-old African Infant. L. G. MacDougall, *Brit. M. J.* 2: 1150, 1954.

Kwashiorkor is believed to be extremely rare under the age of six months. A case is described of a female Jaluo baby of three months, weighing 6 pounds and showing all the signs of kwashiorkor. She was edematous, but had no loss of subcutaneous fat; the skin was pale and the hair depigmented. The liver was enlarged and she had loose, bulky stools. Total serum protein was 4.02 g per 100 ml. The baby had been adequately breast-fed from birth, the breast milk protein concentration being 1.42 per cent on examination. Dried skim milk was given as an additional supplement supplying 8 g of protein daily, but there was no clinical improvement, and the child died. Post-mortem examination showed a terminal pneumonia, an enlarged pale liver which histologically showed generalized fatty change, and some atrophy and fibrosis of the pancreas.—F. E. Hytten

The Vitamin B_6 Content of Milk Products. J. B. Hassinen, G. T. Durbin, and F. W. Bernhart. *J. Nutrition* 53: 249, 1954.

The importance of vitamin B_6 in human nutrition, particularly infant nutrition, has only recently been appreciated. Because cow's milk, most frequently used in the form of sterilized liquids and dried products, is the most important article of diet for nearly all bottle-fed infants, knowledge of the vitamin B_6 content of milk products is of considerable importance. This communication reports: (1) a survey of the vitamin B_6 content of processed milks, including commercial infant formulas; (2) the effect of heat processing necessary for sterilization of canned liquid milk products on vitamin B_6 content; and (3) the heat stability in milk of pyridoxal, pyridoxamine, and pyridoxine.

Sterilized-liquid-milk products were found to contain from 0.10 to 0.35 mg/L and dried-milk products from 0.24 to 0.52 mg/L, which represent 33 to 64 per cent and 69 to 89 per cent of the vitamin B_6 activity of the fresh milk used in their formulation.

The heating process commonly used in industry for sterilization of liquid-milk products causes a considerable decrease in vitamin B_6 content. Decreases in vitamin B_6 of sterilized-liquid-milk products occur not only during autoclaving but continue at a relatively rapid rate for as long as seven days afterwards. Pyridoxine added to sterilized-liquid-milk products is more resistant to heat inactivation than pyridoxal, pyridoxamine, or the naturally occurring vitamin B_6 in milk.—B. Sure

Increase in Intestinal Absorption of Water From Isosomotic Saline Following Pitressin Administration. D. D. Blickenstaff. *Am. J. Physiol.* 179: 471, 1954.

Dogs were prepared with fistulae of the upper jejunum. As these dogs were lying quietly, 0.9% NaCl at a hydrostatic pressure of 15 cm H_2O was introduced, and control values for absorption thus established. Following an injection of pitressin subcutaneously, the tests were repeated. There was a significant increase in water absorption from isosomotic NaCl after pitressin. This finding seems to indicate that intestinal epithelium functions in a manner similar to renal tubules under the influence of pitressin. This action may be pharmacologic and not necessarily physiologic.—M. J. Oppenheimer

The Roentgenologic Appearance of the Small Intestine in Sprue. Long-term Studies with Special Reference to Differential Diagnosis. D. Adlersberg, R. H. Marshak, H. Colcher, S. T. Drachman, A. J. Friedman, and Chun-I Wang. *Gastroenterology* 26: 548, 1954.

A series of forty patients, consisting of thirty females between the ages of 26 and 75, and ten males between the ages of 36 and 67 years were completely studied and the diagnosis of sprue established.

Roentgen examination of the small bowel was carried out and frequent films were taken. Roentgenograms in three patients revealed a normal small bowel pattern. Dilatation was found in 33, segmentation in 34, and scattering in 36. In 36 patients, transit time was between 3 and 5 hours.

Of particular interest was the fact that roentgen evidence of improvement in the sprue pattern occurred in very few of the patients who responded to antianemic or steroid therapy.

This marked discrepancy between the degree of clinical improvement and the roentgen changes reflecting that improvement warrants further investigation.—S. H. LORBER

The Effect on Appetite in Dogs of Pyrogenic Substances in Intravenous Infusion. W. Dale Beamer and J. E. Thomas. *Gastroenterology* 27: 347, 1954.

Healthy dogs were offered an excess amount of meat daily until stabilization of appetite occurred. They were then administered intravenously one of four solutions daily for five days: 500 cc of 0.9 per cent HCl or 250 cc of amino acid mixture (6 to 10 per cent) as pyrogen-free or pyrogen-containing solutions.

Injection of pyrogen-free solutions (saline or amino acids) resulted in no significant change in food intake. Pyrogen-containing saline and pyrogen-containing amino acid solutions both resulted in a decrease in food intake which was statistically significant after the pyrogen-containing amino acid solution.—S. H. LORBER

Relation of Nutrition to Health in Aging Persons. H. D. Chope. *Calif. Med.* 81: 335, 1954.

A follow-up study of 577 California residents over age 50 was carried out with the co-operation of various governmental and state agencies. About two-thirds of the group first studied in 1948 were available for re-evaluation. Mortality data indicate a higher death rate in males than females, and in persons from lower economic levels or with systolic pressures of over 180 mm.

Certain suggestive but inconclusive relationships were also found. The death rate was greater among subjects with a low intake of ascorbic acid, niacin, and vitamin A than in those with a high intake.

The higher the caloric intake, the higher the incidence of digestive tract disease, but the lower the circulatory and nervous system disease incidence.

The incidence of nervous and circulatory system disease bore an apparent inverse relationship to the vitamin A and thiamine intake. Although the number of cases studied is small, and information based on official records is of doubtful value, nevertheless, as this paper suggests, there is an urgent need to carry on further intensive research into the effect of nutrition on the health status of persons over 50 years of age.—S. O. WAIFE

The Interpretation of the Clinical Stigmata of Nutritional Deficiency. R. Passmore. *Proc. Brit. Nutr. Soc.* 13: 105, 1954.

Clinical examinations can contribute greatly to the assessment of nutritional status, if they allow the accurate recording of the incidence of well-defined physical signs and symptoms in a properly selected sample of a population. The incidence of the signs must always be interpreted in relation to a full dietary history and, if possible, correlated with a dietary survey. Only therapeutic trials can establish a nutritional diagnosis with certainty. The absence of definite physical signs of nutritional diseases does not signify that the nutrition of a group is satisfactory. Other methods of assessment, notably dietary surveys, the study of vital statistics, and anthropometric data, are probably more sensitive indices of minor departures from adequate levels of food intake. In the absence of definite physical signs, clinical impressions of nutritional status are subjective and frequently misleading: no direct objective checks between different observers are possible. Nevertheless, in many poor districts in the tropics, and in times of famine and disaster, clinical methods provide the most rapid and reliable of assessments. The Food and Agricultural Organization/World Health Organization Committee's report (FAO/WHO: Joint Expert Committee on Nutrition, 1951) provides an admirable background of our present knowledge of methods of assessment of nutritional status.—B. SURE

Dietary Needs and Weeds in Louisiana. M. F. Gutelius. *South. M. J.* 47: 1196, 1955.

A high carbohydrate diet, low in protein, minerals, and vitamins when compared to the allowances of the Food and Nutrition Board of the National Research Council, is eaten by pediatric patients observed in the clinics of the Charity Hospital of Louisiana at New Orleans. It is possible to improve the diet without increasing its cost by adding a serving of collard greens and substituting one pint of skim milk for eight ounces of whole milk.—M. W. BATES.

Basal Metabolism of Nineteen Children from Two to Ten Years Old. M. W. Lamb and J. M. Michie. *J. Nutrition* 53: 93, 1954.

Since data on the basal metabolism of young children are limited, the authors decided to extend the study of this age group. The results indicate that age alone is an inadequate criterion by which to classify the basal metabolism of children. Basal metabolism varies more uniformly with change in size than in age. The results obtained on these subjects do not conform with the prediction standards developed by Benedict and Dreyer, but agree closely with those of Talbot and Lewis. This agreement can be attributed to the increased basal metabolism and to the complete co-operation of these young subjects.—B. SURE

Human Nutrition and the Sophistication of Foods and Feeding Habits. Some Observations, Implications and Speculations. B. S. Platt. *Brit. M. J.* 1: 179, 1955.

In this lecture, delivered at the annual meeting of the British Medical Association, two main topics are discussed: "the sophistication of foods" by chemical additives used in production, processing and storage and their possible dangers, and the "sophistication of feeding habits," which is largely concerned with possible effects of artificially feeding infants. In the latter, the author considers the possibility of a number of disorders which may be due to a diet short of lactose.

The discussion is superficial and confined to generalities.—F. E. HYTTEN

The Round-Number Barrier in Weight Reduction. L. Kotkin. *New York State M. J.* 55: 98, 1955.

In the author's experience, 31 of 100 patients undergoing weight reduction exhibited a "round-number barrier." That is, treament is terminated by the patient at a round number usually above the desired weight. There is said to be an unconscious factor which motivates cessation of weight loss when, for example, a weight of 150 lb is reached, or a 25-lb weight loss occurs. The author believes that symbolic or magical significance is attached to certain numbers, and this primitive thinking is particularly significant in the obese, whose primitive (oral) mechanisms are so prominent.

This interesting thesis is considerably weakened by the wide definition of a "round number." Thus, weights ending in 0 or 5, and "ten pounds more than the goal-weight agreed upon by the patient and physician," and a level 20 or 25 lb less than the initial starting weight—all are grouped as round-number barriers. It would seem that one-third of any series of numbers would end in 0 or 5, or be 10 lb more or 20 lb less than some numerical figure. Could this phenomenon be solely due to a random distribution?—S. O. WAIFE

Statistical Aspects of Dietary Surveys. B. Woolf. *Proc. Brit. Nutr. Soc.* 13: 82, 1954.

Dietary surveys are widespread and numerous, and have become a regular part of the policy-making apparatus of national and international governing bodies. They are laborious and expensive. There are great variations in methods of investigation and interpretation, and in the scale of operations considered necessary.

From data in the literature of dietary surveys, computations have been made of the errors associated with various phases of the technique of dietary surveys, including the sampling of persons and of period of investigation, the estimation of the foodstuffs eaten, and the calculation of the nutrient content of the diet. Each phase of technique involves random errors, expressible as the coefficient of variation about the mean, and bias, expressible as the difference between the estimated and the calculated true mean. The errors are so large as to make the dietary survey no more than a rather rough instrument. The practical implications of these findings are discussed.—B. SURE

Influence of an Adequate Dietary Protein on the Immediate and Latent Effects of Stilbestrol. S. R. Glasser. *Am. J. Physiol.* 179: 421, 1954.

When stilbestrol was administered, weight was lost. This loss could be attributed largely to decreased food intake. Weight was regained on an adequate protein diet when the hormone was discontinued. If protein was inadequate, weight was not regained. Nitrogen balance was negative during stilbestrol administration, due to reduced food intake. The balance remained negative after the hormone was withdrawn if protein was deficient in the diet. On the other hand, there was a marked nitrogen retention when recovery after stilbestrol took place on an adequate diet. Stilbestrol reduces the loss of liver protein due to caloric restriction. Liver protein was restored during recovery on a basal diet, but losses in liver protein and increase in fat were observed when recovery took place on a protein-free diet.—M. J. OPPENHEIMER

Calcium and Phosphorus Metabolism of Women with Active Tuberculosis. W. D. Brewer, D. C. Cederquist, B. Cole, H. Tobey, M. A. Ohlson, and C. J. Stringer. *J. Am. Dietet. A.* 30: 21, 1954.

The authors report calcium and phosphorus balance data for six women 25 to 39 years of age. The women patients in a sanatorium, had moderately advanced active pulmonary tuberculosis. These data were collected during an earlier study of nitrogen, riboflavin, and thiamine metabolism. The subjects were observed for two weeks on their customary hospital diet. Adjustments were then made in calcium and phosphorus intakes during four 21-day periods by variations in milk intake. In periods 1 and 2, 300

g of milk were consumed daily, and during periods 3 and 4 the milk intake was increased to 1200 g per day. Each period consisted of 14 days of adjustment to the new intake followed by a seven-day balance period. For discussion and presentation, the data for all subjects and periods are arranged according to total daily calcium intake into five groups consuming from 0.5 to 2.49 g/23 hours. Mean calcium intake, mean urinary calcium, mean calcium retention, and number of positive retentions are shown for each of these groups.

Only two patients were found to have positive retentions at calcium intakes below 1.29 g/day. Four of the six retained calcium at intakes ranging from 1.30–1.69 g/day (or daily Ca retention, 0.18 g). Average calcium retention in grams per day varied generally with the daily mean intake of calcium. The regression of calcium retention on intake was significant ($t = 2.14$; $p = 0.05$) and the intake of calcium predicted for equilibrium was 1.22 g daily. This was higher than 0.88 g/day predicted for women 30–39 years of age as reported in earlier studies. The authors believe this apparent increase in requirement for calcium by tuberculous women indicates a disturbance in calcium utilization in pulmonary tuberculosis.

An intake of 1.38 g per day was predicted as required for phosphorus equilibrium. This was not significantly different from values reported in the literature for apparently healthy women.—J. M. SMITH

Problems in Feeding the Tuberculous Patient. H. R. Getz. *J. Am. Dietet. A.* 30: 17, 1954.

The problems of the dietitian feeding tuberculous patients are reviewed. These patients may have lost considerable weight. They have a contagious infection and their nutritive needs include generous quantities of protein, vitamin A, and ascorbic acid.

Evidence for the increased incidence and possible causation of these deficiencies is reviewed. Patients may require 3–4 mg ascorbic acid daily to keep plasma ascorbic acid values anywhere near normal. Night blindness which occurs in many patients cannot be changed by vitamin A therapy; the plasma vitamin A alcohol is low, although the ester form and carotene may be high. The serum albumin is low, while the globulin is increased due to infection.

These deficiencies are caused in part by lowered food intake, due to decreased appetite incident to toxemia from infection. Normal fat handling may be interfered with, and very active peristaltic action in the intestinal tract reduces the time for absorption. It is the opinion of the author, however, that not all of the nutritive failure can be traced to these factors.

The task of the dietitian is to see that the patient receives generous quantities of ascorbic acid, vitamin A, protein, and enough calories to insure weight gain. The ascorbic acid and vitamin A needs are so great as to constitute a medical problem, but patients can take up to 12 oz of orange juice a day without rebelling. One hundred-twenty g protein from meat, milk, eggs, and other foods are recommended, and plenty of butter for vitamin A and unsaturated fatty acids. Liver once a week is desirable. Foods which aggravate the hyperactive peristaltic action, as onions and certain spices, should be eliminated. Appetizing food service and encouragement by the nursing staff are important factors in stimulating patients to eat.

The sanitary problem presented by the tuberculous patient can be solved by proper serving and dish collecting routines and by good washing of dishes.—J. M. SMITH

The Gluten-free Management of Coeliac Disease. D. Lawson. *Proc. Brit. Nutr. Soc.* 13: 75, 1954.

Coeliac disease had for 50 years been one of the most baffling diagnostic and therapeutic problems in clinical pediatrics when, in 1950, the Dutch workers Dicke and Weijers showed that it was associated with an intoxication with the protein fractions of wheat and rye flour. This disease had never been accurately defined and there was always some doubt as to whether it was a single disease. In 75 per cent of the cases the disease begins during the second year of life. A happy infant who has hitherto gained weight steadily throughout a normal infancy gradually, over the course of a few weeks, goes off his food, ceases to gain weight, and perhaps vomits occasionally, while the stools are noticed by an observant mother to become progressively paler, bulkier, softer, and more offensive. In most cases the child loses his happy disposition and becomes difficult, cantankerous, and impossible to feed or to amuse.

The relative simplicity of the gluten-free diet gave Dr. Sheldon high hopes that by this means the success already achieved in hospital might be sustained after discharge. The diet has been applied with full success to 64 cases; these have since expanded into 120, which are now the subjects of a prolonged follow-up study.—B. SURE

Unsaturated Fatty Acid Oxidation Catalyzed by Hematin Compounds. A. L. Tappel. *Arch. Biochem. & Biophys.* 50: 473, 1954.

In earlier studies, it has been suggested that the tocopherols protect unsaturated fatty acids, vitamin A, and carotene from oxidative destruction. This paper reports the inhibition by antioxidants, methylene blue, and other substances having *in vivo* vitamin E activity, of unsaturated fatty acid oxidation catalyzed by hemin, hemoglobin, and cytochrome c.

The oxidation of colloidal linoleate suspensions catalyzed by 10^{-6} M hemoglobin or cytochrome c was inhibited by 10^{-4} M concentrations of α-tocopherol. Propyl gallate was similarly effective, and nordihydro-

guaiaretic acid and butylated hydroxyanisole were ten times as potent as α-tocopherol in inhibiting the catalyzed oxidations of linoleate. Vitamin A and carotene were not oxidized in the presence of 10^{-4} M hemin, but were co-oxidized in the presence of a hemin-catalyzed oleic acid oxidation. The catalyzed oleic acid oxidation and the vitamin A and carotene co-oxidation were both inhibited by α-tocopherol.

Methylene blue and α-tocopherol strongly inhibited the hemin-catalyzed oxidation of oleic acid, cod liver oil and linseed oil, but methylene blue markedly increased the autoxidation of oleic acid, and had very little effect on the autoxidations of cod liver oil and linseed oil. Several substances having *in vivo* vitamin E activity were found to be potent inhibitors of the hematin-catalyzed oleic acid oxidations. The author suggests that inhibition by methylene blue, thionine, and other substances having *in vivo* vitamin E activity, may be due to their combining with the hematin catalysts, probably with the iron, in contrast to the tocopherols and phenolic antioxidants which can destroy fat peroxides, a necessary precursor of the oxidative reactions.—M. K. Horwitt

Army Operational Rations. H. G. Smith. *Proc. Brit. Nutr. Soc.* 13: 45, 1954.

Under normal conditions, the soldier is fed according to a standard ration scale based on normal civilian feeding habits. Items of fresh food, such as bread, frozen meat, vegetables, and fruit, are provided, and all the necessary cooking equipment is available for preparing the meals. When, however, supply conditions become difficult, as during wartime when troops are situated far from their home bases, tinned equivalents of the fresh items are brought into issue. Such substitution may be applied to part or to the whole of the ration, and it is continued until fresh items again become available through the development of local resources and the arrival of ships with frozen meat and other foods.

The conditions which operational rations should satisfy may be summarized briefly as follows: (1) The ration should be nutritively adequate, attractive, and sufficiently varied in composition to ensure general acceptability when consumed over a fair period of time. (2) The items in the ration should be such that appetizing meals can be prepared by the soldiers themselves, who are probably unskilled in the art of cooking, without the aid of normal cooking equipment. (3) The ration should be in a compact and convenient form, suitable for rapid distribution and use during special operations such as assault landings, or on long-range patrols. (4) The packaging should be adequate to withstand very rough handling without damage to the contents, and the weight of packing should be reduced to a minimum consistent with efficiency. (5) The ration packs must withstand long storage in any climatic conditions without chemical or microbial deterioration.—B. Sure

Study of the Immunizing Ability in Experimental Pyridoxine Deficiency Due to Desoxypyridoxine and to Diet. L. B. Donnasibilla and M. Cortesi. *Acta vitaminol.* 8: 269, 1954.

Pyridoxine deficiency, however produced or protracted (up to 55 days of antivitamin administration and 5 months of pyridoxine-deficient diet), was found to have no effect on active immunizing ability in rats. In animals rendered deficient by the administration of the antivitamin, vaccine stimulation resulted in a production of antibody levels entirely comparable to that obtained in controls. The natural immunity in desoxypyridoxine-induced deficiency in no way differed from that observed in animals on a pyridoxine-deficient diet. Neither type of deficiency affected the complement activity of the serum, but in both the hemolysin titer was elevated.—C.-J. Howell

Nutritional Requirements in Cold Climates. K. Rodahl. *J. Nutrition* 53: 575, 1954.

Although it is generally recognized that proper feeding is an essential factor in maintaining normal health in a cold environment, the actual nutritional requirements under the great variety of arctic conditions are by no means firmly established. It has been suggested by several authors that the arctic calorie requirements would be of the order of 5500 to 6000 calories per man per day both for men engaged in field operations, as well as for troops on garrison duties in the arctic or subarctic. On the other hand, a dietary survey carried out among European trappers in Greenland, 1939–40, showed that the white trappers maintained their body weight on an average gross consumption per man of 3000 calories per day.

A series of nutritional surveys has been carried out among two groups of whites (airmen and infantry soldiers) in Alaska during the four seasons of the year from 1950 to 1952. Simultaneously, similar studies were made among four groups of Eskimos for comparison.

Individual food weighings showed an average daily calorie consumption per man of 3000 in the air force group and 3200 in the infantry group. The average calorie expenditure for the four seasons was estimated to be about 2800 calories per man per day on the basis of time activity data. Under these conditions, no appreciable weight change occurred, and the subjects remained in excellent health throughout the period of study. It is concluded that the calorie requirements of the average man engaged in activities of similar magnitude and under similar climatic conditions as those of the subjects studied, would be in the order of approximately 3000 to 3500 calories per man per day at any season of the year. In adult male Eskimos at four different locations in Alaska, an average daily consumption of about 3100 calories was sufficient to maintain body weight with an estimated daily energy expenditure of roughly 2700 calories throughout the year. These findings are in agreement

with previous findings among Eskimos and trappers in Greenland.

The percentage of calories furnished by protein, fat, and carbohydrate in the United States troops living in Alaska is not significantly different from that reported for United States troops eating a garrison ration in temperate or tropic climates. In the air force group, however, there was an increased consumption of fat in the winter.

The consumption of minerals was higher than the recommended allowances for temperate climates.

The data presented do not indicate that the human vitamin requirements are significantly higher in arctic or subarctic environments than in temperate climates. In the case of vitamin C, arctic travelers, trappers and Eskimos may subsist on ascorbic acid intakes of less than 15 mg daily without showing clinical evidence of vitamin C deficiency.—B. SURE

Collective Acute Lead Poisoning. H. Bénard, A. Gadjos, M. Gadjos-Török, and P. Rambert. *Sem. Hôp. Paris* 16: 785, 1953.

This is a report of a chemical and biologic study of an episode of collective acute lead poisoning, occurring among the crew of a tanker and apparently caused by the *ingestion of a wine* containing 4.5 mg of lead per litre.

Chemically, this poisoning is characterized by gastrointestinal signs, by severe impairment of the general condition, and by a more or less marked anemia. These chemical signs disappear rapidly following withdrawal of the toxic substance.

The biologic signs (plumbemia, presence of punctuate red blood cells, hypercoproporphynuria, free globular hyperprotoporphyria) persist for a longer period.

These biologic stigmata are not observed with equal frequency. The disturbances of porphyrin metabolism appear to be the most constant.—H. GOUNELLE

Skim Milk Powders and Experimental Rat Caries. F. J. McClure and J. E. Folk. *Proc. Soc. Exper. Biol. & Med.* 83: 21, 1953.

In previous studies, the authors had shown that heat processing of cereal foods predisposed towards a type of dental caries in the rat. Rats receiving diets in which the major source of protein was skim milk had a significantly higher incidence of surface caries when the skim milk was heat-treated. Certain other factors in the processing of skim milk may also play a part in its cariogenic activity.—L. W. KINSELL

Phosphorus Metabolism in Unsaturated Fatty Acid-Deficient Rats. P. D. Klein and R. M. Johnson. *J. Biol. Chem.* 211: 103, 1954.

The aspects of metabolism which are dependent upon the unsaturated fatty acids for their proper function were studied through the observation of alterations in phosphorus metabolism associated with either glycolytic or respiratory mechanisms. In fat deficiency the decreased uptake of P^{32} by the acid-soluble organic P fraction appeared to be related to a dissociation of oxidation from phosphorylation accompanying the oxidation of Krebs cycle intermediates by the DPN-linked dehydrogenases. Since no differences were found between the normal and fat-deficient groups with respect to their anaerobic glycolytic ability, it was concluded that the defect was restricted to oxidative phosphorylation.—M. K. HORWITT